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Binding of Hepatic Nuclear Proteins From Obese and Lean Zucker Rats to the Upstream Region of Malic Enzyme Gene.

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**BINDING OF HEPATIC NUCLEAR PROTEINS
FROM OBESE AND LEAN ZUCKER RATS TO THE UPSTREAM REGION
OF MALIC ENZYME GENE**

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

The Department of Microbiology

by

Yibing Wang

M.D., Shandong Medical University, 1987

M.S., Shandong Medical University, 1990

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DEDICATION

I wish to dedicate this work to my wife, Min. It has been through her constant love, support, and understanding that I have found the strength and stability to meet the challenges I have faced. I do not believe that I could possibly have accomplished my graduate studies without her constant encouragement.

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ABSTRACT

Adrenal glucocorticoids are essential for the development of all forms of experimental obesity. In the obese (*fa/fa*) Zucker rats, adrenalectomy prevents the further development of obesity, and glucocorticoid replacement restores the obese phenotype. We have hypothesized that this dependence on glucocorticoids is due to differences in transcriptional regulation of a group of genes that are normally regulated by glucocorticoids. We have chosen to study the DNA fragments from the upstream region of the malic enzyme (ME) gene, which is positively regulated by glucocorticoids. Hepatic nuclear extracts from lean and obese Zucker rats were used in band shift studies to look for differences in binding of nuclear proteins to the DNA. All of the upstream DNA fragments bind more obese nuclear protein than lean nuclear protein. Adrenalectomy decreases the amount of obese nuclear protein bound to the 222 bp fragment to the same level as the lean nuclear protein. Treatment of the nuclear proteins with alkaline phosphatase altered gel retardation patterns and suggests that phosphorylation state of the nuclear proteins is important for DNA binding. The hepatic nuclear extracts were also analyzed in 2- dimensional SDS PAGE. Two proteins were present in lean nuclear extract but absent from obese nuclear extract. The proteins were N-terminal blocked and could not be used for N-terminal sequencing. These data suggest that changes in nuclear proteins may be involved in the altered transcription of this glucocorticoid regulated gene.

Our study suggests that differences between lean and obese Zucker rats in nuclear proteins binding to the upstream regions of ME gene may

reflect changes in phosphorylation state and differential effects of glucocorticoids.

REVIEW OF LITERATURE

Obesity affects more humans than any other health problem. One fourth of the United States adult population was reported to be more than 20% above normal weight in 1981 (1). After a decade, more than three in ten adult Americans now fall in this category (2), and many of these are grossly obese (3). Indeed, epidemiological studies showed that the prevalence of obesity is increasing from generation to generation (4).

Although the mechanisms leading to obesity status are complex and diverse, familial studies clearly indicate the influence of genes on metabolic regulation (5-7). In an adoption study of human obesity recently performed by Stunkard et al. (5) with 540 adult adoptees, a strong relation between the weight class of the adoptees and the body-mass index of their biologic parents was observed, whereas there was no relation between the weight class of the adoptees and the body-mass index of their adoptive parents. Cumulative distributions of the body-mass index of parents showed similar results. This indicates that genetic influences have an important role in determining human fatness in adults, whereas the family environment alone has no apparent effect (5). To date, there are several genes associated and/or linked with human obesity (8).

Animal models of genetic obesity

Animal models of genetic obesity, especially single-gene mutation obese animals, are particularly useful for the study of obesity. They can be maintained in standard inbred backgrounds, and appropriate matings of the animals can give rise to predictable numbers of unaffected and affected

animals, all of which have the same genetic background, differing only by the presence of a single mutant gene or a small region of the chromosome containing the mutant gene (9).

In 1905, genetically determined obesity was first described in the yellow mouse by L. Cuenot (10). Since then, animal models have been used to study the metabolic and behavioral changes that lead to obesity. The results have been applied to the human obesity caused by single gene mutations (8).

More than five mouse mutations and at least one rat mutation causing obesity have been intensively studied and are encoded on five different mouse and rat chromosomes (8). These rodent obesity genes code for regions that have human homologous equivalents, which are located on five different human chromosomes (Table 1) (11). These animal models cover a wide range of changes in the manifestation of obesity coupled with varying degrees of diabetes and provide investigators models with which to advance our understanding of the causes and development of obesity-diabetes conditions (9). To date, three of the mouse genes (*a*, *ob* and *fat*) have been cloned (12-14).

The yellow mouse

The agouti locus (*a*) is located on mouse chromosome 2. It differentially regulates the synthesis of eumelanin (black or brown) and phaeomelanin (yellow) pigment granules by the melanocytes within the hair follicle (15). Since the original description of the yellow (*A^y*) mouse (10), a number of additional alleles have appeared at the agouti locus (12, 16). Different combinations of alleles result in an array of different phenotypes,

Table 1. Possible synteny between rodent obesity genes and human chromosome regions

Locus	Transmission	Rodent chromosome	Human homologous region
Diabetes (<i>db</i>)*	Recessive	4	1p31-pter
Obese (<i>ob</i>)*	Recessive	6	7q31
Tubby (<i>tub</i>)*	Recessive	7	11p15.1
Fat (<i>fat</i>)*	Recessive	8	16q22-24
Yellow (<i>A^y</i>)*	Dominant	2	20q11.2
Zucker (<i>fa</i>)**	Recessive	5	1p31-pter

Adapted from references 11, 15 and 17.

*, from mice.

**, from rats.

ranging from subtle changes in coat color from the wild type, to drastic changes in the distribution of pigmentation in different regions of the animal, especially across the dorso-ventral surface. The lethal yellow (A^Y) mouse has an all-yellow phenotype in the heterozygous condition (15, 18), the black-and-tan (a^t) mouse has an all-black dorsum and an all-yellow ventrum (19), while the nonagouti (a) mouse has a predominantly black phenotype (16) and extreme nonagouti (a^e) mouse has a completely black phenotype (20). Silver and coworkers did a series of experiments to demonstrate that the a locus functions within the microenvironment of the hair follicle (16, 21-24).

The homozygous dominant yellow mutation causes death in very early stages in development, around the time of implantation possibly due to a delay in giant cell differentiation, preventing the embryo's attachment to the endometrial cells that line the uterus (25-28).

Pleiotropic effects of the dominant agouti mutations include pronounced obesity (29-32) and increased susceptibility to diabetes (33). Heston and coworkers were able to demonstrate that the lethal yellow heterozygotes have a tendency to develop a variety of spontaneous and induced neoplasms including pulmonary tumors, mammary-gland tumors, and hepatomas (34-38). In addition, it was reported that the lethal yellow heterozygotes exhibit a systemic stimulation in body growth and reduced fertility in females (39).

A breakthrough came when a radiation-induced inversion mutation which contains DNA breakpoints in the *limb deformity* (ld) and agouti loci was reported in 1990 (40). A DNA probe from the ld region was used to successfully clone a region of DNA that maps to the agouti locus (40). Two

years later, the agouti gene was cloned and characterized (12). It was shown to encode a 131 amino acid protein. The gene is normally expressed predominantly in neonatal skin (12). The dominant yellow (A^y) mutation is associated with translocation and overexpression of the gene in most tissues of the adult mouse (12, 18). In 1994, a human homologue of the agouti gene was mapped to chromosome 20q11.2 and cloned. The agouti gene and the protein that it encodes are now under extensive study. A recent report showed that the agouti protein is a high-affinity antagonist of the melanocyte-stimulating hormone (MSH) receptor and blocks α -MSH stimulation of adenylyl cyclase, which is the effector through which α -MSH induces eumelanin synthesis (41). It was reasoned that the obesity caused by ectopic expression of agouti in the lethal yellow (A^y) mouse may be due to the inhibition of melanocortin receptor(s) outside the hair follicle (41).

The obese and diabetes mice

The *ob* mouse was first described by Ingalls et al. in 1950 (42). It inherits its obesity as an autosomal recessive mutation on chromosome 6 (43). The earliest detectable change in preobese *ob/ob* mice is an impairment in thermogenesis (44-45). During the dynamic phase of the obesity, hyperinsulinemia and hyperphagia increase with the progressive appearance of glucose intolerance, fasting hyperglycemia, and resistance to exogenous insulin (46). However the obesity of *ob/ob* mice is not dependent on the hyperphagia (47-49).

The diabetes (*db/db*) mutation on chromosome 4 (43) originally arose on the C57BL/KsJ background. It is also an autosomal recessive gene. The *ob* and *db* mutation cause similar if not identical diabetes-obesity syndromes

when maintained in the same inbred background (43). The only comparative data that distinguish these two mutations from each other are the results of the well-known parabiosis experiments (cross circulation studies) performed by Coleman (50-51). In these experiments capillary anastomoses (exchanging 2-3% of cardiac output) were made between three sets of mice. In the first set (*ob* to wild-type), it was found that obese mice lost weight, indicating that *ob* is deficient for a circulation factor that suppresses appetite. In the second set (*db* to wild-type), it was noted that the normal mice starved to death while the *db* mouse survived, suggesting that the *db* mouse is resistant to a circulating appetite suppressant and it secondarily overproduces that factor. The third set used *ob* and *db* mice and it was found that the *ob* mice starved to death, suggesting that *db* mice are resistant to the same factor that is absent or defect in *ob* mice (11).

With the development of the techniques of positional cloning, the mouse *ob* gene and its human homologue were recently cloned by J. M. Friedman's group (13). First, in 1991, they positioned *ob* on mouse chromosome 6 and mapped the *ob* gene between two restriction-fragment length polymorphism (RFLP) markers, D6Rck13 and Pax4 (52-54). Then they cloned the DNA in the region of Pax4 and D6Rck13, using both probes to start the construction of a physical map in the region of *ob* (13). Finally, using the method of exon trapping, they were able to isolate genes from a 650-kb interval that was indicated in previous genetic studies to contain the *ob* gene (13, 55). By screening putative exons for the presence of corresponding RNA from a variety of tissues using northern blots and reverse-transcription PCR, they found 6 genes, one of which, 2G7, was

hybridized to a northern blot of mouse tissues. A 4.5-kb RNA that exists only in white adipose tissue was detected and the 2G7 was used to screen a mouse adipose tissue cDNA library and the isolated clones sequenced (13). Their results showed a 167 amino acid open reading frame followed by a long 3'-untranslated sequence (13). The predicted amino acid sequence is 84% identical between human and mouse and has some features of a secreted protein (13). They suggested that the *ob* gene product may function as part of a signaling pathway from adipose tissue that acts to regulate the size of the body fat depot. In a severely obese *ob/ob* mouse line studied by Zhang et al., a nonsense mutation in codon 105 of the mouse gene has occurred and it expresses a 20-fold increase in adipose tissue *ob* mRNA. Another *ob* mutant mouse strain studied by the same group does not show any evidence for the expression of the gene (13), indicating the importance of the *ob* product in the maintenance of the non-obese status.

Recently, three articles reported the *in vivo* studies of the effect of the so called OB protein in mice, which were carried out by three independent research groups (56-58). In these studies, the recombinant mouse OB protein was expressed in bacterial systems and purified to nearly homogeneity. Administration of the OB protein intraperitoneally (56-58), intravenously, or intracerebroventricularly (58) all resulted in a remarkable decrease in the body weight of *ob/ob* mice compared with that of the lean animals. These results prove that the OB protein is the "circulating appetite suppressant" suggested by Coleman in his parabiosis experiments mentioned above (50-51). The most obvious effect of the OB protein is the reduction of the animal's appetites. But food intake alone does not seem to

account for the overall weight loss induced by the OB protein because the OB-treated mice lost 50% more weight than the untreated mice even when they were on the same diets (57). Pelleymounter et al. were able to show that besides the reduction of the animals' appetites, the OB-treated mice increased their total activity to the level of the lean animals and the metabolic indices in them were normalized (56). These findings indicate that the OB protein also increases the energy expenditure in these animals. Furthermore, also consistent with Coleman's parabiosis experiments, the OB protein has no effect on *db/db* mice, which seem to have a defect in the receptor for the OB protein (57-58). The OB receptor seems to exist at least in the brain because direct injection of small dose of OB protein into the brains of mice resulted in similar effects that were caused by much higher doses when given intravenously (58). However, whether the OB receptor may exist elsewhere and what exactly happens after the binding of the OB protein to its receptor remain to be elucidated.

The fat and tubby mice

The *fat* and *tub* mice were first described by Coleman and Eicher in 1990 (9), although they were originally found as spontaneous mutations at The Jackson Laboratory in early 1970's. Breeding tests indicated that these obesity conditions were both caused by autosomal recessive mutations. The development of obesity syndrome in *tubby* and *fat* is slow compared to the phenotypically similar *ob* and *db* mice. Problems in glucose homeostasis are mild and only transient. Hyperglycemia is only seen in homozygous *fat* males between the ages of 7-11 weeks whereas *fat* females and *tubby* mutants of both sexes remain normoglycemic or slightly hypoglycemic (9). Both of the

homozygous *tub* and *fat* mice are infertile, but not sterile, and can produce litters when the animals are mated before severe obesity develops (5-8 weeks for *fat/fat* and 12 weeks for *tub/tub*).

Although mice with a genotype of *fat/fat* develop obesity at a slower rate than homozygous *ob* or *db* mice, they still can become two (male) or three (female) times heavier than wild-type litter mates (9, 14). Most of the excess weight in the *fat* mutants results from increased weight of body fat that distributes to fat stores throughout the body. This is different from the pear-shaped body conformation caused by the confinement of body fat to the axial and inguinal regions in the *ob* and *db* mutants.

The obesity syndromes in the *fat/fat* rats include increased rate of weight gain, high blood sugar concentration, hyperproinsulinemia, and abnormal pancreatic morphology. Interestingly, these conditions remain mild and diabetes was not present when the *fat/fat* mutation was placed on the BKs inbred background. This is in contrast with the changes from mild to severe diabetes observed when either the *ob* or *db* mutation was placed on the BKs inbred background.

The most striking feature of *fat* mutants is the pronounced hyperinsulinemia (14) which becomes apparent as early as 4 weeks of age, when obesity is not detectable (9). This symptom was observed in both the BKs background and the HRS/J background, in which the *fat* mutant was originally identified.

In 1994, the *fat* mutation was mapped to mouse chromosome 8 by Paigen and Coleman (59). In 1995, Naggert et al. were able to link the *fat* gene to the vicinity of carboxypeptidase E (Cpe or Cph-1) by screening for

recombinants in progeny of *+fat* matings (14). Cpe is an exopeptidase involved in the processing of prohormones including proinsulin (60). Naggert et al. showed that the hyperinsulinemia in *fat/fat* mice is a consequence of abnormal Cpe expression which results in abnormal proinsulin processing. They also demonstrated that the mutant Cpe gene contains a single missense mutation (Ser202Pro), which abolishes CPE enzymatic activity when expressed in a baculovirus system. All these data strongly support that *fat* is a mutation at the Cpe locus. But it is still not known whether that *fat* phenotype is a direct consequence of faulty processing of proinsulin or some other hormone because *fat* mice are not always hyperglycemic. Since the expression of Cpe is not limited to pancreatic islets, Naggert et al. proposed that the development of obesity may be a more direct consequence of abnormal processing of some other neuroendocrine prohormone (e.g. neuropeptide Y, enkephalins, proopiomelanocortin and oxytocin-vasopressin), and not simply insulin (14). The fact that the *fat/fat* mice are functionally infertile also supports the suggestion that there are defects in the neuroendocrine axis.

Tubby mice are phenotypically very similar to *fat* mice except for slower development of obesity (The phenotype cannot be recognized until 9-12 weeks of age) and the existence of sexual dimorphisms with respect to rate of weight gain, plasma insulin concentration, and pancreatic morphology in *tub* mice. The average weight at 24 weeks is 46.4 ± 0.9 g in *tub/tub* males compared to only 38.6 ± 1.1 g in females. Both sexes develop mild hyperglycemia associated with hyperinsulinemia. Histological examination of the pancreas showed relatively normal islet morphology in both sexes when

compared to normal littermates. Some enlargement of the islets occurred at 12 weeks of age in males and 24 weeks in females. Degranulation of the β cells was seen only in the late stages, indicating that insulin synthesis and secretion in *tub/tub* mice can be sustained at levels sufficient to maintain near normal β -cell granulation (9). The *tub* gene has been located on chromosome 7 near the Hbb (hemoglobin beta-chain complex) locus and related studies are still underway.

The Zucker rats

The obese Zucker (*fa/fa*) rat was first described in 1961 as a spontaneous mutation in a cross between the Merck Stock M and Sherman rats (61). The obese phenotype is transmitted as an autosomal recessive trait and the homozygous animals suffer the consequences of genetic obesity, which closely resembles human juvenile onset obesity (62-64).

Zucker fatty rats eat large meals compared with the food intake of their lean litter-mates (47-55, 65-69). However hyperphagia is not the only responsible factor because pair-feeding obese rats with lean ones does not prevent the development of the obesity (69-70) and food restriction before weaning failed to stop the hypertrophy and hyperplasia of adipose tissue depots (71).

The concentration of both circulating cholesterol and triglycerides in the obese Zucker rats is very high (61, 69, 72). This hyperlipidemia is characterized by increases in all lipoprotein classes with marked elevations in very low density lipoproteins (VLDL) (73). The pathogenesis of the hyperlipidemia in obese Zucker rats is not completely understood. It was suggested that increased production of lipoproteins is the major cause (74).

In the liver of obese Zucker rats there is an increase in lipogenesis with a concomitant decrease in fatty acid oxidation (75-76). The activity of lipoprotein lipase, which hydrolyzes plasma triglycerides, is elevated in white adipose tissue and reduced in brown adipose and muscle as early as the first or second week of life (77-78).

Hypertrophy and hyperplasia of the β -cells in obese Zucker rats results in a major hypersecretion of insulin. The insulin levels in obese Zucker rats are above those of the lean rats as early as 3 weeks of age (65, 79). Fasting insulin levels were increased by 10-fold in *fa/fa* rats compared with lean Zucker rats (80). Postprandial insulin levels were even higher in obese Zucker rats (69). Despite this increase in plasma insulin levels, obese Zucker rats had higher plasma glucose levels than lean Zucker rats (80). This is because obese Zucker rats also develop insulin resistance, which has been well documented (69, 74, 80-83). The life span of obese Zucker rats can be divided into two stages based on insulin resistance. In the first stage, there is a pancreatic oversecretion of insulin with a reduced hepatic clearance of the hormone (84), while a general state of insulin resistance is present in the later stage (69). Both *in vitro* and *in vivo* experiments have demonstrated the insulin status in obese Zucker rats. Decreased insulin binding to plasma membranes has been observed in isolated liver, muscle and adipose cells (69), and it has been shown that hepatic glucose production in obese Zucker rats was not inhibited by insulin, indicating that gluconeogenesis and lipogenesis are insulin resistant in liver (69). Insulin resistance was initially thought to be caused by decreased insulin binding because of the observation of decreased insulin receptor numbers in the

obese Zucker rats (85). It was later realized that this decreased binding was not sufficient to explain the overall state of resistance because it has been shown that food intake restriction or pair-feeding can restore the levels of insulin to normal in adult rats (76). It was reported that insulin-stimulated protein kinase activity, a postreceptor event, was decreased in both heart and liver tissue from obese Zucker rats (86). These studies suggest that insulin resistance in obese Zucker rats involves both receptor and postreceptor abnormalities as will be discussed later.

The thermogenic capacity of brown adipose tissue is significantly reduced in obese mice or rats when compared with that in lean ones although the obese ones exhibit increased brown fat mass (87-88). It has been shown that the brown fat metabolism of the obese Zucker rats does not increase as much in response to maximum doses of the beta agonist isoproterenol as in the lean rats (89) and the maximum activities of several brown fat oxidative enzymes are lower in obese Zucker rats (78).

G. E. Truett and coworkers mapped *fa* between two molecular markers, *lfa* and *Glut-1*, which also flank *db* on mouse chromosome 4 and are located on rat chromosome 5. Their results placed *fa* on rat chromosome 5 and suggested that *db* and *fa* are mutations in homologous genes (90). Both the *fa* and *db* gene appear to have a human counterpart on chromosome 1p31 (29).

In the experiments carried out in 1981 by R. B. S. Harris using tube-feeding induced obese rats (91), the obese rats appeared to produce a blood-borne factor that has a very mild effect on food intake and caused the selective depletion of body fat, indicating that this factor is probably anti-

lipogenic. The parabiosis experiments between *fa/fa* and wild-type rats performed later by the same group (66) showed that the fatty rat exerts the same effect as do *db* mice. Interestingly, it has been shown that ventral medial hypothalamus (VMH) lesioned rats also behave similarly to *db* mice and *fa* rats in parabiosis experiments (67). All these results indicate that the blood-borne factor in rats is very likely the rat homologue of mouse *ob* gene product and this factor is not functioning properly in these *fa/fa* rats. Not surprisingly, it was recently found that the *ob* mRNA level in *fa/fa* rats was 10 fold higher than in their lean litter mates (Abstract in press, X. Lin and D. A. York).

Glucocorticoids and obesity

Since the first description and isolation of glucocorticoid hormones more than half a century ago (92), their roles in development, metabolism and the response to stress have been intensively studied.

Glucocorticoids are secreted from the zona fasciculata of the adrenal cortex after being synthesized from cholesterol by a series of reactions (93). The major glucocorticoid in man is cortisol and in rat, corticosterone. About 95% of the glucocorticoids in the circulation are bound to plasma proteins, primarily corticosteroid-binding globulin. The secretion of glucocorticoids is regulated by adrenocorticotrophic hormone (ACTH, corticotropin). ACTH is produced by the pituitary gland and is regulated by corticotropin releasing factor (CRF), which is in turn produced by the hypothalamus. By feedback regulation, the glucocorticoids inhibit ACTH production and secretion, probably at both the hypothalamic and pituitary levels (94). Because the glucocorticoids are lipid-soluble, they readily cross the plasma membrane

and combine with the glucocorticoid receptor, the first steroid receptor discovered (95).

As signals to cope with stress, glucocorticoids have effects on a wide range of tissues and metabolic pathways, involving stimulation of many physiologic, metabolic, cardiovascular, and other functions (93). The metabolic effects of glucocorticoids lead to the propensity of hyperglycemia and nitrogen wasting. In the liver, the glucocorticoids increase glucose production because of the induction of gluconeogenic enzymes, permissive effects on the actions of glucagon and epinephrine, and increased availability of substrate. Glucocorticoids induce breakdown of both protein and nucleic acid and inhibit protein synthesis in muscle, making muscle the major source of amino acids. In adipose tissue the glucocorticoids stimulate lipolysis, promote the lipolytic effect of epinephrine, and increase the production of lactic acid. Glucocorticoids and insulin generally have antagonistic effects whereas the actions of a number of other hormones may be amplified by glucocorticoids. It has been suggested that glucocorticoids may be required by the adrenal medulla to produce epinephrine, which has β -adrenergic activity (93).

Glucocorticoids have an important function in regulating body weight (93). Both acute and chronic administration of corticosterone can induce obesity in normal mice (92). The cause for this response is probably increased food consumption, and circulating insulin as well as the decrease of thermogenesis in brown adipose tissue (96-98).

Circulating glucocorticoid levels are increased in the *ob/ob* and *db/db* mice (99-100). However most studies have found normal levels of

corticosterone in the obese Zucker (fatty) rat (65, 101-105), although some studies have shown marginally increased circulating levels of corticosterone (101-102). Studies in rats with VMH lesions also showed normal corticosterone concentration (27). These results indicate, as will be discussed below, that there is an increased responsiveness to glucocorticoids in the experimental animal obesities (46, 106).

The development of all the experimental obesity models can be prevented or suppressed by adrenalectomy and restored by replacements of glucocorticoids (46). It occurs in hypothalamic obesity after VMH and PVN lesions (107), in dietary obesity after feeding high fat diets to certain strains of rat (108), in older rats that become obese on "cafeteria" diets (109) and in the genetic obese animals such as *ob/ob* (98, 110-113), *db/db* (114), and *A^y/a* (115) mice and *fa/fa* rat (116-120).

An increased sensitivity and responsiveness to glucocorticoids appears to be characteristic of the experimental animal obesities (46, 106). This altered responsiveness and sensitivity were widely observed in the studies with replacement doses of glucocorticoids given to adrenalectomized lean and obese animals (110, 119, 121-122).

Most of the abnormalities that are associated with the development of obesity in animal models, especially those that are involved in carbohydrate and protein metabolism, have been shown to be influenced by the restorative effects of adrenalectomy. These abnormalities include body weight (88, 123), fat deposition (124), fat cell size (120), muscle weight (111), energetic efficiency (88, 123-124), food intake (88, 125), meal pattern (125), thyroid function (112), islet cell distribution (114), insulin concentration

(115, 124), insulin response (113), glucose tolerance (96), protein synthesis (126), lipoprotein lipase (115), lipogenesis (117), brown adipose tissue function (127), body temperature (128), mRNA level of malic enzyme and GAPDH (118), etc. Although the *fa/fa* rat may have marginally increased circulating levels of corticosterone (101-102) this cannot account for the glucocorticoid dependence since replacement of adrenalectomized obese rats with glucocorticoids will restore obesity whereas identical treatment of adrenalectomized lean rats has no such effect.

Although adrenalectomy has a wide-ranging restorative effects as mentioned above, it does not restore body composition and serum insulin levels precisely to those seen in lean rats because some of the changes are already irreversible. This is well demonstrated by observation of serum insulin. The serum insulin falls to low levels after adrenalectomy (112) but still remains slightly elevated when compared to the lean animals. This has been reasoned to result from the existing hyperplasia and hypertrophy of the islet tissue because serum insulin levels of the weaned *fa/fa* rat have been found to stay at the lean levels if the adrenalectomy is performed prior to weaning (117). The reduction of serum insulin level in the obese animals after adrenalectomy probably results from a decreased activity of the parasympathetic nervous system and the enteroinsular axis and an increased activity of the sympathetic nervous system that links to the pancreas (117). Therefore it seems that adrenalectomy normalizes autonomic balance, islet anatomy (114), and glucose metabolism (96, 129).

In a study performed by Langley and York (130), young obese *fa/fa* rats were treated with RU-38486, a type II glucocorticoid receptor antagonist,

for 15 days. Analysis of body composition changes showed that RU-38486 effectively reversed the obesity. It stopped fat deposition in obese rats but increased protein deposition to the level of lean-vehicle rats. RU-38486 also prevented the development of hyperphagia and reduced gross energetic efficiency in the obese rats but had little effect on lean rats. The study also showed that mitochondrial GDP binding was increased in brown adipose tissue of obese rats but was reduced in lean rats by RU-38486 treatment. RU-38486 also reduced the elevated activity of hippocampal glycerophosphate dehydrogenase, a glucocorticoid-responsive enzyme, of obese rats to the level of lean rats. Their results suggested that abnormal activity of glucocorticoid receptors or abnormal cellular responsiveness to corticosterone receptor complexes may be important in the development of obesity in the *fa/fa* rat.

A transgenic mouse with impaired corticosteroid receptor function was created by partially knocking out gene expression with type II Glucocorticoid receptor (5q31-32) antisense RNA (131). The transgenic animals had increased fat deposition and a body mass that by about 6 months of age was twice as large as that of controls. An elevated body fat content was observed despite the fact that transgenic animals ate about 15% less than the normal mice. Increased serum concentrations of corticosterone and ACTH, a similar feature seen in obese *fa/fa* rats, were also seen in these animals. These results clearly indicated the important role that glucocorticoids play in the development of obesity. However, they argue against all other experimental data, which showed the positive effect of the glucocorticoid receptor in the development of obesity, although the mechanism is still unknown.

Insulin and the development of obesity

Discovered over 70 years ago, insulin is the most important secretory product of the pancreatic beta cell and plays a central role in the regulation of many key metabolic processes (132). Hyperinsulinism, insulin resistance, and decreased number of insulin receptors are characteristic of obesity in both human and experimental animals (133). As has been mentioned above, fasting insulin levels were increased by as much as 10-fold in obese Zucker rats compared with lean Zucker rats (80). Postprandial insulin levels were even higher in obese Zucker rats.

In contrast to glucocorticoids, which are steroid hormones, insulin is a polypeptide hormone (134) produced by the β -cells of the pancreas in response to nutritional, neural and endocrine stimuli. The most important physiological regulator of insulin release is glucose (135). Amino acids, especially the essential amino acids leucine, arginine, and lysine, have been shown to stimulate insulin release in the absence of glucose (136). However, various lipids and their metabolites appear to have only minor effects on insulin release (137). The pancreatic hormone glucagon has a stimulatory effect on insulin release (138), while somatostatin inhibits insulin release (139). Other hormones, such as glucocorticoids, prolactin, growth hormone, placental lactogen, and the sex hormones, have also been found to stimulate insulin secretion (140). It was reported that the adrenergic neurotransmitters, norepinephrine and epinephrine, suppress insulin release and parasympathomimetic agents stimulate insulin release (141). *In vivo* studies of beta-cell secretory function have demonstrated that insulin is released in a pulsatile manner (142).

The major site of insulin metabolism is liver (143), which extracts half of the insulin delivered to it. The first step in degradation of the hormone appears to be binding to receptors on the hepatocyte surface. After insulin is internalized, at least some of them has been shown to serve as the substrate for degradation (143). Other sites of insulin metabolism include kidney, adipose tissue, blood cells, and placenta (144).

Although insulin exerts regulatory or growth effects on almost every cell of the body, the most important tissues for insulin action are muscle, liver, and adipose tissue (132). Insulin activates the transport systems and enzymes involved in utilization and storage of glucose, amino acids, and fatty acids, and inhibits gluconeogenesis, glycogenolysis, lipolysis, and protein breakdown (145).

Insulin released into blood binds to the α subunit of its receptor in its target tissues and activates the receptor tyrosine kinase. This, in turn, initiates the cellular processes that regulate organismal glucose homeostasis by activation of existing transport systems, recruitment of intracellular proteins such as glucose transporters (GLUT-4) from intracellular storage vesicles to the plasma membrane (146-147), and covalent modification of pre-existing enzymes by phosphorylation or dephosphorylation such as pyruvate dehydrogenase, acetyl-CoA carboxylase, triacylglycerol lipase, phosphorylase kinase, and glycogen synthase and phosphorylase (148). On a slightly slower time frame (i.e., several minutes to a few hours) than for nutrient transport, insulin also regulates transcription of specific genes and new protein synthesis. It has been known that insulin stimulates the synthesis of pyruvate kinase, malic enzyme, and glucokinase and inhibits

that of phosphoenolpyruvate carboxy kinase (PEPCK), carbamoyl phosphate synthetase I, and fructose-1,6-bisphosphatase (145, 149).

Phosphorylation of the glucocorticoid receptor and insulin receptor

Although the discovery of protein phosphorylation as a mechanism by which extracellular signals regulate cellular functions was dated as early as the late 1950s (150), it has been only a few years since protein phosphorylation/dephosphorylation began to be recognized as a critical mechanism in signal transduction used by numerous growth factors and hormones, including glucocorticoids and insulin (151-152). The phosphorylation of nuclear proteins has been widely observed in the regulation of gene transcription (153). The C-terminal domain (CTD) of the large subunit of RNA polymerase II has multiple repeats that can be phosphorylated at multiple sites (154). It has been shown that the hyperphosphorylated form is the major form in transcribing polymerase II complexes (155) and that only the un-phosphorylated form of polymerase II can enter the initiation pathway. It was proposed that phosphorylation of this subunit may be needed to trigger conversion of an initiation complex into an elongation complex (156) although the true nature of the CTD protein kinase is unknown. Pulverer et al. were able to demonstrate that the DNA binding of c-Jun is negatively regulated at one set of sites by phosphorylation and phosphorylation at another set of sites increases transactivation activity (157). Phosphorylation is also reported to be able to modulate nuclear translocation and other properties of transcription factors such as the interaction between factors when bound to neighboring sites in a promoter or enhancer (158). However, the identification of transcription factors which

direct gene transcription as a result of posttranslational modification has been difficult to demonstrate.

There have been reports of changes in the activity of a protein phosphatase (glycogen synthase phosphatase) (159-160) in hepatocytes from obese Zucker rats. Furthermore, M. D. Houslay's studies showed that ligands which enhanced G_i -2 phosphorylation in intact hepatocytes from lean Zucker rats failed to do so in hepatocytes from obese animals. They suggested that this defect in guanine-nucleotide-mediated " G_i function" seen in obese Zucker rats may be due to an inactivating phosphorylation of αG_i -2 (161).

Glucocorticoid receptor

Like other steroid receptors, the glucocorticoid receptor consists of a ligand binding domain, a DNA binding domain, and a poorly conserved N-terminal region which is considered to be a transcriptional modulation domain (162-163).

In the absence of the glucocorticoid hormone, a monomer of the glucocorticoid receptor, two molecules of heat shock protein (HSP90), and several other heat shock proteins interact with each other and form an aporeceptor complex (164). The complex is in dynamic equilibrium with two unliganded, non-HSP90-bound receptor forms, namely an unliganded inactive receptor that can neither bind to DNA nor enhance transcription and an unliganded "active" receptor that can bind to DNA and enhance transcription (165). Hormone binding leads to activation of the receptor and dissociation of HSP90 (166), which is a substrate-specific chaperone (68, 164).

Proper interaction of the glucocorticoid receptor with HSP90 is essential for efficient ligand binding and response (167-169). It was recently suggested that steroid receptors, including glucocorticoid receptors, may require heat shock protein 70 (HSP70, or DnaK) (168) and DnaJ like proteins (170-171), both molecular chaperones, for assembly and maintenance of the aporeceptor in the absence of ligand and for proper folding of the activated receptor after ligand binding (165).

Upon binding of the glucocorticoid to the receptor, the activated receptor-hormone complex then translocates from the cytoplasm to the nucleus, probably with the help of certain proteins that promote nuclear import (172). The receptor-hormone complex binds to specific glucocorticoid response element (GRE) (173-175) on the DNA of target genes which allows binding of transcription factors and RNA polymerase to initiate or inhibit transcription. The DNA binding domain of the receptor consists of 2 cysteine "zinc fingers" which interact with specific bases in the GRE (173). GR will only fully regulate gene transcription when it binds to the GRE in the form of a dimer (176).

Glucocorticoid receptor is basally phosphorylated and undergoes hyperphosphorylation after hormone-induced activation (177). In the mouse glucocorticoid receptor, seven phosphorylation sites have been identified by Bodwell et al. in 1991 (178). Single mutations at all seven sites and combinations of substitutions displayed levels of hormone-induced reporter gene expression that are similar to wild type receptors (179). Thus it seems that phosphorylation of the GR at the seven identified sites is not a major determinant in GR mediated transcription activation. Therefore

phosphorylation of various components of the GR signal transduction pathway, and not necessarily the receptor itself, may influence its transcriptional regulation activities (180).

Phosphorylation can increase the negative charge and acidity of one or more regions of a protein, influencing its interactions with other proteins or with DNA. Thus hyper- and hypophosphorylation at the same time in different regions or at different time in the same region of steroid receptor molecules might serve as a mechanism for differential transcription regulation of certain genes (181). Furthermore, the fact that there are so many phosphorylation sites affected by different kinases/phosphatases and signal transduction pathways suggests that phosphorylation is involved in the regulation of multiple receptor functions (181).

Insulin receptor

The insulin receptor is located on the surface of most mammalian cells, including both the classic insulin target tissues (liver, muscle, and adipose tissue) and nonclassic target tissues (blood cells, brain, etc.). The number of insulin receptors ranges from less than 100 per cell to more than 200,000 per cell, the higher concentrations primarily being on adipocytes and hepatocytes. Any insulin analogue that binds to the receptor exerts a biological effect in proportion to its affinity for the receptor. The insulin receptor is a transmembrane glycoprotein with a molecular weight of approximately 350 to 440 kDa. The receptor is a heterotetramer composed of two α -subunits (135 kDa) and two β -subunits (95 kDa) linked by disulfide bonds (182-183). The α subunits are located entirely outside the cell. The two subunits are covalently linked to each other by disulfide bonds. There

are 735 amino acids in the α subunits which contain the insulin binding sites. The β subunits are transmembrane proteins and are composed of a 193 amino acid extracellular domain, a 23 amino acid alpha-helical transmembrane domain, and a 402 amino acid intracellular domain that possesses an insulin-regulated tyrosine protein kinase activity (182-183).

In 1981, Kasuga et al. first reported that the insulin receptor contains a tyrosine kinase activity (184). This finding was later confirmed by cDNA cloning, which revealed that the insulin receptor belongs to the family of tyrosine kinase receptor family (185). The receptor tyrosine kinases have been divided into three groups (186): the epidermal growth factor (EGF) receptor family which are monomeric with two clusters of cysteine-rich regions in the extracellular domain and the major autophosphorylation sites in the C terminus (187), the platelet-derived growth factor/colony-stimulating factor 1 (PDGF/CSF-1) receptor family which have an immunoglobulin-like distribution of cysteine residues in the external domain and a stretch of ~100 amino acids which interrupts the intracellular kinase domain (188), and the insulin receptor family which has a single cluster of cysteine residues in the extracellular α subunits and a kinase domain in the β subunits that resembles the kinase domain of the prototype tyrosine kinase (189).

The tyrosine kinase activity of the insulin receptor is essential for all known early and late cellular responses evoked by insulin (190). In the unoccupied state, the tyrosine kinase activity intrinsic to the β subunit of the insulin receptor is inhibited by the α subunit. This inhibition can be released by insulin binding or removal of the α subunits using proteolytic enzymes (191). It has been shown that insulin binding induces a propagated

conformational change through the transmembrane domain in the β subunit of the receptor (192-193).

The most critical intracellular portion of the insulin receptor β subunit required for normal receptor function is the consensus amino acid sequence encoding an ATP binding domain marked by a distinctive glycine-rich motif (Gly-X-Gly-X-X-Gly) followed by a lysine residue 12 amino acids downstream. This lysine residue is absolutely required for insulin action since substitution with any other amino acid invariably blocks autophosphorylation and kinase activity and all biological responses (194).

There are a total of seven phosphorylation sites in the intracellular domain of the β subunit. Activation of the insulin receptor by insulin binding leads to the autophosphorylation of the three tyrosines in the regulatory region in the intracellular domain of the β subunit (195). This, in turn, results in a greatly increased kinase activity (196). Using mutation analysis, Sun et al. were able to show that another autophosphorylated tyrosine residue residing in the juxtamembrane region of the receptor is important for the phosphorylation of endogenous receptor substrates (197). Furthermore, the insulin receptor is under regulation by serine and threonine phosphorylation (184).

In 1985, White et al., using antiphosphotyrosine antibodies that recognize proteins based on the presence of the phosphotyrosine side chain independent of the primary sequence of the protein and ^{32}P -labeled hepatoma cells, identified the major intracellular substrate of the insulin receptor, IRS-1 (originally named pp185) (198). Phosphorylation of IRS-1 was found to reach to its maximal level within seconds after the cells were

exposed to insulin. The dose-response curve was similar to that of receptor autophosphorylation, suggesting that it represents the endogenous substrate for the insulin receptor kinase. Subsequently, IRS-1 has been found in most cell types. The amino acid sequences of IRS-1 are 98% homologous between human and mouse, and 90% homologous between human and rat.

IRS-1 has approximately 35 to 50 potential Ser/Thr phosphorylation sites. It is strongly serine-phosphorylated even in the basal state. The phosphorylation level is further increased after insulin stimulation (199). IRS-1 also contains 14 potential tyrosine phosphorylation sites, six in Tyr-Met X-Met motifs and three in Tyr-X-X-Met motifs (197). The fact that these sites are identical in rat, mouse, and humans suggests that they have important functions (200). It has been shown that, following autophosphorylation of the tyrosine residues, a number of cellular enzymes involved in signal transmission could bind reversibly to Tyr-Met X-Met or Tyr-X-X-Met sites in some oncogene products, and PDGF and EGF receptors (200). The proteins that bind to these phosphorylation motifs have been found to contain special recognition domains termed SH2 (*src* homology 2) domains (201), which are structural elements in many proteins that bind with high affinity to phosphotyrosine residues in activated tyrosine kinases and participate in cellular signaling (200). SH2 domains have been found in some oncogene products (such as *src*, *abl*, and related tyrosine kinases) (202), some structural proteins, several enzymes (including the phosphatidylinositol 3'-kinase, the phospholipase C γ , and the GTPase-activating protein of ras) (203), and a recently discovered class of adapter proteins represented by the protein Grb-2 (204).

Phosphorylated IRS-1 has been demonstrated to bind strongly to phosphatidylinositol 3'-kinase (PI-3-Kinase) (197), which phosphorylates the lipid phosphatidylinositol. This binding has been shown to be between the tyrosine-phosphorylated Tyr-Met-X-Met motifs of IRS-1 and the SH2 domains in the p85 subunit of the PI-3-Kinase (205). Therefore it has been speculated that IRS-1 might function as a mediator in insulin signal transmission by binding to certain SH2 domain-containing proteins during insulin stimulation and regulating the associated catalytic activities that mediate the insulin response (190). It has also been suggested that the PI-3-Kinase is involved in the insulin-induced glucose transporter translocation in adipocytes and skeletal muscle (206)

By a mechanism not yet clear, the initial tyrosine cascade of insulin action triggers a series of serine and threonine phosphorylation and dephosphorylation reactions (190). Insulin stimulation leads to the activation of a variety of serine protein kinases, including the MAP kinases, Raf-1 kinase, acetyl-CoA carboxylase kinase, ATP-citrate lyase kinase, casein kinase II, and the ribosomal S6 kinases (207).

Recent reports have linked insulin receptor signaling to the Ras activation pathway (208-209). The observations that ras mutation or antibodies against ras abolished insulin action clearly indicated that ras is an integral component of certain insulin signaling pathways (210-211). It has been shown that all ras-related proteins bind guanine nucleotides (GTP and GDP) and possess intrinsic GTPase activity (212). The ras protein has been suggested to serve as a biological switch that is "on" when GTP is bound and "off" when the GTP is hydrolyzed to GDP (213). The GTPase activity of

ras is regulated by two accessory proteins, guanine nucleotide-releasing protein (GNRP) which catalyzes the dissociation of GDP from ras, and GTPase-activating protein (GAP) which catalyzes hydrolysis of GTP to control the GTP/GDP ratio. A recently cloned molecule Grb-2, which contains one SH2 domain and two SH3 domains, has been suggested to serve as an adaptor in the signal pathway between the EGF receptor and ras (209). The activation of Ras requires autophosphorylated receptors such as PDGF, EGF, and IRS1. The Grb-2 binds to the IRS-1 and to a GNRP called mSos (mammalian son of sevenless), linking insulin action to the ras pathway (208-209, 214-218).

The key component of the Ras pathway is the so called mitogen-activated protein kinases (MAP kinases), or extracellular signal regulated kinases (ERKs), which were first described by Ray and Sturgill in 1987 (219). The Ras pathway activated by tyrosine phosphorylation (220) leads to activation of c-Raf, the serine/threonine protein kinase and protooncogene product, which in turn activates additional serine/threonine kinases including MAP kinase kinase/ERK kinase (MEK) (221). The activation of MAP kinase kinase leads to the activation of serine/threonine kinases or "switch kinases" such as the MAP-kinases in a cascade of kinase activity leading to alterations in gene transcription and other cellular changes (153, 222). The MAP-kinase cascade is triggered not only by several tyrosine kinase receptors, but also by G protein coupled receptors (190).

The substrates for the MAP-kinases include cytoplasmic enzymes such as p90^{rsk} (the 90 kDa form of ribosomal protein S6 kinase), MAPKAP-

kinase 2 (MAP kinase-activated protein kinase 2), and phospholipase A2 and a series of transcription factors such as c-Jun, c-Myc, and c-Myb (207). In order to elucidate the signal transduction pathway of insulin action on gene expression, beginning in late 1980's, researchers have been attempting to define cis-acting sequences that mediate the effect of insulin on gene transcription and work backward to define the mechanisms by which the trans-acting factors that interact with these sequences are regulated (223-225).

With the help of mutation analysis of a number of insulin responsive genes and functional analysis in several transfection systems, insulin-responsive elements (IREs) have been delineated in the 5' flanking region of several genes including those for phosphoenolpyruvate carboxykinase (PEPCK), c-fos, amylase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucagon, insulin-like growth factor binding protein-1 (IGFBP-1) and malic enzyme (223-230). However, to date, none of the proteins that bind to these IREs have been characterized and none of the known transcription factors has been found to bind to the IREs (190). A distinctive feature of the IREs is the lack of a consensus sequence and both positive IREs and negative IREs exist. The IREs of the PEPCK gene and IGFBP-1 gene mediate negative regulation by insulin (225, 229, 231), the IRE of the amylase gene mediates positive regulation by insulin (226), while there are two IREs on the malic enzyme gene, one positive, the other negative (230). One or more proteins have been shown to be able to bind to the above mentioned IREs (225-227, 229) during gel shift assays. None of the proteins responsible for the different bands formed in these gel shift

assays has been characterized. Nevertheless insulin has been shown to increase the amount of protein that binds to the IREs for growth hormone and GAPDH. This increase apparently resulted from a phosphorylation event that increases the affinity of the protein for its IRE (224, 232-233). Therefore, phosphorylation/dephosphorylation of transcription factors may serve as a common mechanism by which insulin regulates the transcription of a variety of genes.

Malic enzyme and its gene

Malic enzyme (ME; E.C. 1.1.1.40), the activity of which was originally identified in pigeon liver extracts by Ochoa et al. (234), catalyzes the NADP-dependent oxidative decarboxylation of malate (235):



The pyruvate formed in this reaction readily diffuses into mitochondria, where it is carboxylated to oxaloacetate by pyruvate carboxylase. The NADPH provides reducing power required for fatty acid synthesis.

ME is a tetramer of four structurally identical subunits and is widely distributed (236). It has been grouped with other enzymes which are important in lipogenesis, including the hexose-monophosphate shunt dehydrogenases, ATP-citrate lyase, and fatty-acid synthetase. The level of ME strongly correlates with fatty acid synthesis and is regulated by complex hormonal (237-239) and dietary (240-243) interactions. The ME gene is transcribed into two mRNAs of 3.1 kilobase (kb) and 2.1 kb, respectively. Although both mRNAs can be effectively translated into the functional protein, they are differentially expressed in a tissue-specific manner; in liver, the 2.1 kb form is preferentially expressed while the other form is expressed

in other mammalian tissues. However, there is no evidence up to now for a selective transcriptional regulation, and it has been suggested that different tissue-specific polyadenylation factors are involved (244).

Bray and York (118) reported that the levels of mRNA for ME and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were increased in liver and adipose tissue of young obese *fa/fa* rats. Hepatic ME and GAPDH mRNA was reduced by adrenalectomy of *fa/fa* rats and showed a dose and time dependent increase after corticosterone replacement, while glucocorticoid replacement had much smaller effects on ME and GAPDH mRNA in lean rats.

The coding nucleotide sequence of rat hepatic ME mRNA as well as a 882 base pair (bp) 5'-flanking region of the ME gene have been cloned and sequenced by V. M. Nikodem's group in late 1980's. Consistent with the enzyme's wide tissue distribution, the structural organization of the ME promoter resembles promoter regions of several eukaryotic constitutive or "housekeeping" genes (245).

Deletion analyses of the ME gene promoter region showed that sequences +1 to -41 are sufficient to initiate expression (245). Inclusion of information up to -177 is necessary for maximal promoter activity. Further upstream region (up to -882) contains the binding sites for many nuclear proteins and transcription factors (Table 2, Figure 1), such as thyroid hormone receptor (TRE), insulin related proteins (IRE), Jun, Fos (AP-1), Sp1, HNF3 α , and glucocorticoid receptor (GRE). A TATA box (245, usually located 20 to 30 bp upstream from cap sites, lies at -626 to -621 (Figure 1).

Table 2. Protein binding sites on ME gene upstream region

Binding sites	Positions	Sequence	References
IRE-I	-693 to -687	TGTTTTG	230
HNF3 α binding site	-696 to -687	TATTGTTTTG	246
TATA	-626 to -621	TATAAA	245
GRE	-501 to -487	CAGGAAAATTGTTCT	247
TRE	-281 to -266	TTGGGGTTAGGGGAGG	248
IRE-II	-162 to -169	CCCGCCC	230
Sp1	-177 to -168	GCCCCGCCCC	249
AP-1	-132 to -126	TGACTCA	249

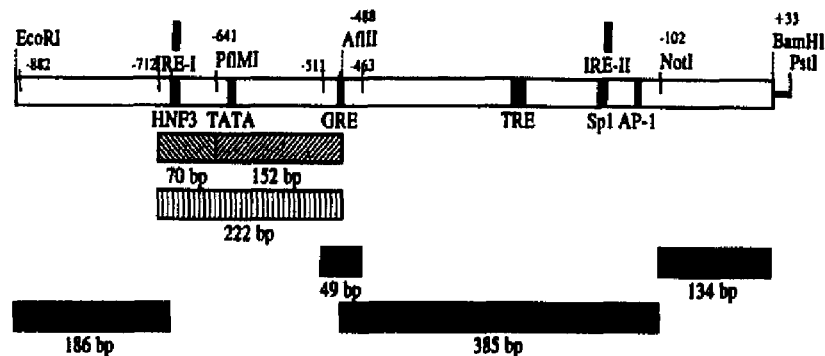


Figure 1. The map of the upstream region of ME gene. The 925 bp upstream region of the ME gene is shown (open bar). Sites used to generate the DNA fragments to be used in the band shift assays are indicated above the open bar with their positions. DNA fragments are shown as either shaded or hatched bars below the open bar and their sizes indicated below each fragment. Sequences that bind nuclear proteins are shown as solid boxes in the open bar. Restriction sites mentioned in the text are also indicated above the open bar.

Therefore it is of interest to investigate the binding of the hepatic nuclear proteins from obese and lean Zucker rats to this region of the ME gene in order to gain knowledge about the mechanism of the involvement of glucocorticoids and insulin in the development of obesity.

Objectives

In order to further our understanding of the mechanisms that lead to obesity, and to verify the hypothesis that the increased glucocorticoid responsiveness in obese animals is caused by altered transcription factors, this dissertation research was undertaken to examine the binding of hepatic nuclear proteins from obese and lean Zucker rats to glucocorticoid regulated genes such as ME. The initial study on the SDS PAGE and 2-D PAGE patterns of the nuclear proteins was also carried out. The specific objectives are to:

1. Subclone the 882 bp DNA fragment of the upstream region of ME gene into pUC19 and restrict this insert into fragments of the appropriate sizes to be used in band shift assays.
2. Perform gel retardation assays using nuclear proteins from obese and lean Zucker rat liver and different fragments from the upstream region of the ME gene to identify those regions of the upstream DNA that are involved in the regulation of the rate of transcription in the rat and, hence, are regulated differently in the lean and obese animals. This would enable us to identify the site-specific DNA binding proteins in nuclear extracts from lean and obese rats.
3. Carry out the same type of experiments using the identified DNA fragment(s) and nuclear extracts from lean and obese animals that have

been adrenalectomized. These binding studies were done in the presence and absence of glucocorticoid hormones, and the results were compared to those of the normal lean and obese animals. This will make it possible to identify the protein that is involved in the glucocorticoid control of ME gene.

4. Investigate the effect of dephosphorylation and phosphorylation on the formation of the DNA-protein complexes.
5. Analyze the nuclear proteins present in the specific DNA-proteins complexes.
6. Analyze the hepatic nuclear extracts using SDS PAGE and 2-D PAGE.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacteria strains and plasmid vectors used in this study are described in Table 3.

Media and growth conditions

All bacteria strains were routinely subcultured in LB medium (250) at 37°C, Liquid cultures were aerated vigorously. 1.5% (w/v) agar was added for solid media. Ampicillin when used was added to media at 100 µg/ml.

Chemicals and reagents

pUC19 DNA, 10 X ligation buffer, T4 DNA ligase, DNA polymerase I large (Klenow) fragment, Vent_R[®] (exo⁻) DNA polymerase, and all restriction enzymes used in this study were purchased from New England Biolab; Ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, ethidium bromide, nonidet P-40, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropylthio-α-D-galactoside (IPTG), magnesium acetate, potassium chloride (KCl), triton X-100, calf intestine alkaline phosphatase (CIAP), protein kinase A (PKA), myosin, phosphorylase A, catalase, actin, carbonic anhydrase, lysozyme and ampicillin were purchased from Sigma Chemical Company; protein kinase C (PKC) was purchased from Boehringer Mannheim; AmpliTaq DNA Polymerase was purchased from Perkin Elmer Cetus; SeaKem GTG agarose was purchased from FMC BioProducts; Tris-HCl, Glycine, Magnesium chloride (MgCl₂), and 10 X TBE buffer were purchased

Table 3. Plasmids and host cells

	Genotype	Reference
Host Cells:		
JM83	<i>araΔ(lac-proAB) rpsI (strA) (φ80lacZΔM15)</i>	251
INVαF'	F' <i>endA1 recA1 hsdR17 (r_k⁻, m_k⁻) supE44 thi-1 gyrA96 relA1 φ80lacZ ΔM15 Δ(lacZYA-argF) U169</i>	252
Plasmids:		
pME882-CAT	CAT	245
pCR TM II	Ap ^r	253-254
pUC19	Ap ^r	255

from AMRESCO; Magic Miniprep Kit was purchased from Promega; 10 X Polymerase Chain Reaction (PCR) buffer was purchased from Invitrogen; Ammonium acetate was purchased from Fisher Scientific; Sodium dodecyl sulfate (SDS) and glycerol were purchased from United States Biochemicals; 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxythymidine 5'-triphosphate (dTTP), deoxyribonucleoside triphosphates (dNTPs), and poly(dI-dC)•poly(dI-dC) were purchased from Pharmacia; [α - 32 P]dATP or [α - 32 P]dGTP was purchased from DuPont; Sucrose was purchased from GIBCO BRL; Anti-glucocorticoid monoclonal antibody (BuGR2) was purchased from Affinity BioReagent; Anti-HNF3 α antiserum was a gift from Dr. James Darnell.

Subcloning of a 882-bp upstream region of ME gene

The plasmid pME882-CAT (Figure 2) which contains the ME 5' flanking region was provided by V. M. Nikodem (245). After digestion of pME882-CAT with restriction enzymes EcoRI and PstI, the reaction mixture was loaded onto a 0.7% agarose gel and the gel was run at 40 V overnight in 1 X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 8.0). After the gel was stained with ethidium bromide for 10 min and washed with water for 10 min, a band of 942-bp was seen under UV light (Figure 3). This band, which contains the 882-bp upstream region of ME gene, was excised from the gel, cut into small pieces (dimension approximately 2 mm x 2 mm x 5 mm), and packed into a 0.5 ml inner elution tube which had been filled with 1 X TBE buffer. The DNA fragment was eluted using a Hoefer Scientific Instruments GE 200 Eluter at 200 V, for 3 hours. The eluted DNA was then added to a

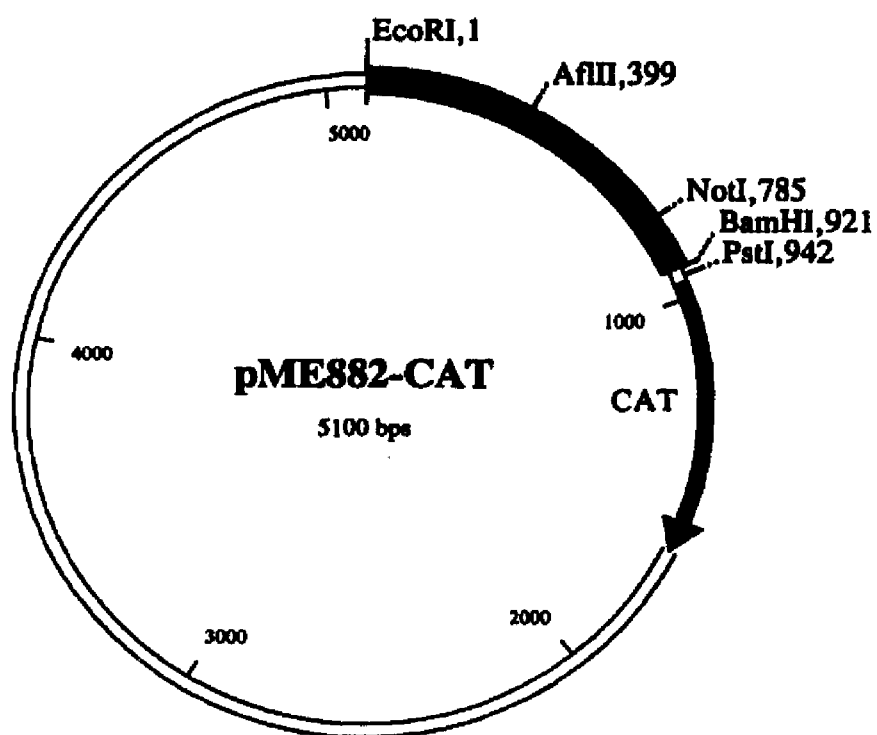


Figure 2. The map of pME882-CAT. The CAT gene is indicated by a solid arrow. The 925 bp upstream region of ME gene carried by this plasmid is shown as a shaded bar. Restriction sites are indicated and numbered according to their distance to the **EcoRI** site.



Figure 3. Generation of the 942 bp DNA fragment. Digestion of pME882-CAT with EcoRI and PstI gave rise to a 942 bp DNA fragment that contains the 925 bp upstream region of the ME gene. Lane 1 shows the 1 kb DNA ladder used as a DNA size marker. Lane 2 shows the digestion reaction resolved on a 0.7% agarose gel. The position of the 942 bp DNA fragment in lane 2 is indicated by an arrow on the right.

centricon (Amicon) with a cut-off of 10,000 MW, centrifuged at 3500 rpm in an International Equipment Company HN-SH centrifuge at 4°C for 3 hours. 100 ng of the 942-bp DNA and 100 ng of pUC19 DNA, which had been digested with EcoRI and PstI, were added into a ligation reaction which contained 1 X ligation buffer and 400 units of T4 DNA ligase in a total volume of 20 µl. The ligation reaction was carried out at 16°C overnight. The recombinant DNA was then transformed into competent JM83 cells following the procedures described by Maniatis et al. (256) The transfected cells which contained recombinant plasmid were identified as white colonies by plating dilutions of the transformation on L-agar, into which 100µg/ml ampicillin had been added, in the presence of the chromogenic substrate X-gal and the gratuitous inducer of β-galactosidase IPTG. The recombinant plasmid was purified using Magic Miniprep Kit. After digestion of the plasmid with restriction enzymes AflII-NotI and NotI-PstI, the insert was confirmed by loading the restriction digestion mixture onto a 5% polyacrylamide gel electrophoresis (PAGE) and run at 25 V, overnight with 1 X TBE as running buffer (Figure 4). The recombinant plasmid was named pME925 (Figure 5).

Two other DNA fragments, representing -881 to -696 and -712 to -491 of the ME gene, were generated by polymerase chain reaction (PCR) amplification using gel purified, EcoRI digested pME925 as template. The primers used in the PCR reactions were designed using a primer designer program, Primer 2 (Scientific & Educational Software) and were synthesized by the GeneLab at the School of Veterinary Medicine Louisiana State University. The names and sequences of the primers are shown in Table 4. The PCR reactions were performed in a Perkin Elmer Cetus DNA thermal

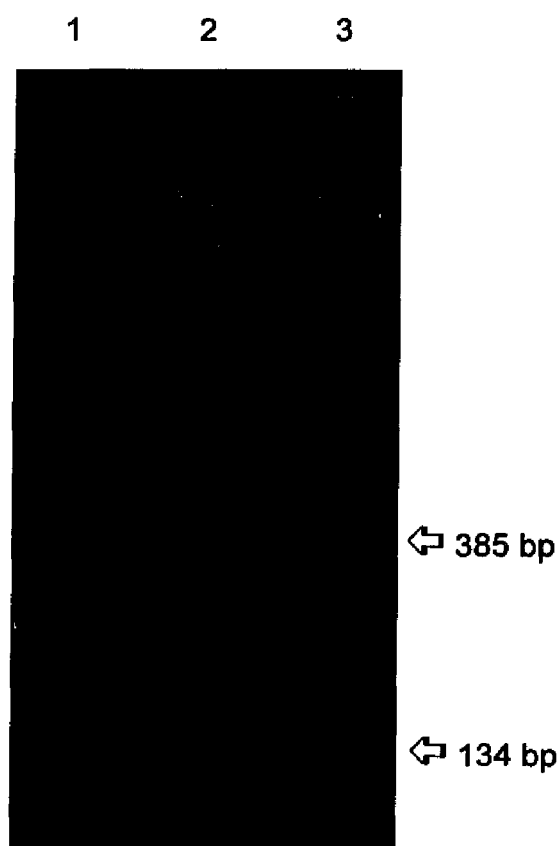


Figure 4. Generation of the 134 bp and 385 bp DNA fragments. Digestions of the plasmid pME925 with NotI-BamHI and AflII-NotI gave rise to a 134 bp and 385 bp DNA fragments, respectively. Lane 1 shows the 100 bp DNA ladder used as a DNA size marker. Lane 2 (NotI/BamHI) and 3 (AflII/NotI) contain the digestion reactions resolved on a 5% PAGE. Positions of the 134 bp and 385 bp DNA fragments are indicated by the arrows on the right.

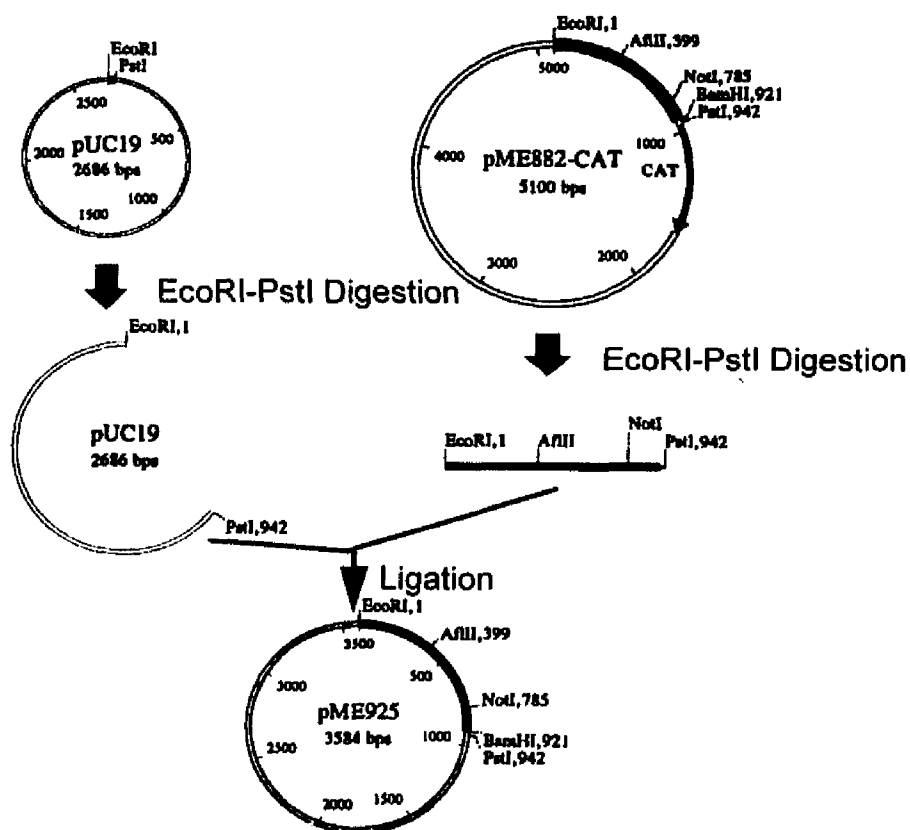


Figure 5. Cloning of a 945 bp **EcoRI**-**PstI** DNA fragment into **pUC19** vector. The plasmid **pME882-CAT** was digested using **EcoRI** and **PstI** and a 942 bp DNA fragment containing ME upstream region was isolated. The DNA fragment was then ligated into **EcoRI**-**PstI** digested **pUC19** vector.

Table 4. Primers used in PCR reactions

Names	Sequences
MECLN1A	CGCATAGCCCAGAAG
MECLN1B	ACAGCCAAGCAGCCT
MECLN2A	GAAGGCTGCTTGGCTGTATTG
MECLN2B	CAATTTTCCTGAGCGTTCTGG
ME49A	CCAGAACGCTCAGGAA
ME49B	CAGTGGCCAGTCCTAT

cycler. Each PCR reaction consisted of 90 ng of the template DNA, 1 X PCR buffer, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 1 μ M of each of the two primers, and 1 unit of the thermostable Taq DNA polymerase in a total volume of 50 μ l. The primers MECLN1A and MECLN1B were used in the PCR reaction to generate the 186 bp DNA fragment and the primers MECLN2A and MECLN2B were used in the PCR reaction to generate the 222 bp DNA fragment. Because the non-template dependent activity of the thermostable polymerase used in PCR adds single deoxyadenosines to the 3'-end of all duplex molecules, the resulting 186 bp and 222 bp DNA fragments were ligated into the specifically designed plasmid vector Invitrogen pCRTMII, which provides single 3' T-overhangs at the insertion site. The thermocycles used are described in Table 5. The recombinant plasmid generated using primers MECLN1A and MECLN1B was designated pME186 and the one generated using primers MECLN2A and MECLN2B, pME222 (Figure 6).

End labeling target DNA

Labeled DNA fragments used in the band shift assays were prepared by digestion of the plasmids pME925, pME186 or pME222 with different restriction enzymes and filling in with either [α -³²P]dATP or [α -³²P]dGTP depending on the sequences of the 3-recessive ends of the fragments.

The plasmid pME925 was digested with the restriction enzymes AflII-NotI or NotI-BamHI, the plasmid pME186 was digested with EcoRI, and the plasmid pME222 was digested with EcoRI and EcoRI-PfIMI.

The labeling reactions were carried out with 1 μ g digested plasmid DNA, 0.5 mM dNTPs as needed, 0.825 μ M (50 μ Ci) [α -³²P]dATP or

Table 5. Thermocycles used for PCR

Names of the products	Primers used	Thermocycles	Number of Cycles	Delay
ME186	MECLN1A	94°C, 1 min	25	72°C
	MECLN1B	47°C, 1 min		10 min
		72°C, 1 min		
ME222	MECLN2A	94°C, 1 min	25	72°C
	MECLN2B	50°C, 1 min		10 min
		72°C, 1 min		
ME49	ME49A	94°C, 1 min	25	
	ME49B	41°C, 1 min		
		72°C, 3 sec		

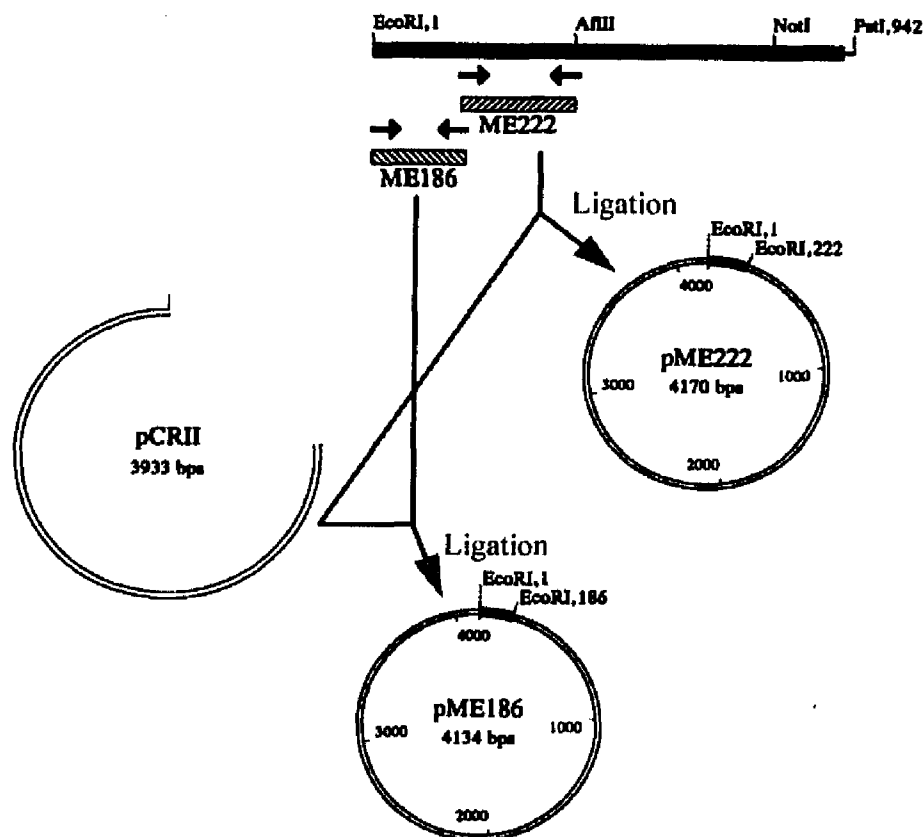


Figure 6. Construction of the plasmids pME186 and pME222. A 186 bp and a 222 bp DNA fragments were obtained using PCR. The 942 bp DNA fragment was used as the template. The DNA fragments were then ligated into the pCRII vector which provides single 3' T-overhangs at the insertion site. The solid bar represents the 925 bp upstream region of the ME gene. The restriction sites were indicated above the solid bar. The positions of the two DNA fragments relative to the 925 bp upstream region of ME gene are shown with the hatched bars below the solid bar.

[α - 32 P]dGTP and 5 units Klenow fragment in a total volume of 20 μ l. After incubation at 37°C for 15 min, the labeling mixtures were loaded onto a 5% polyacrylamide gel and electrophoresed at 200V, room temperature for 40 to 60 min. And the bands were visualized by staining with ethidium bromide as described above. The bands were then cut out of the gel with a lancet and transferred into a microcentrifuge tube containing 200 μ l elution buffer which is composed of 0.5 M ammonium acetate, 1 mM EDTA, 10 mM magnesium acetate, and 0.1% SDS, and shaken at 225 rpm in a 37°C incubator overnight. The elution buffer containing the DNA fragments was then transferred to another microcentrifuge tube and the gel slices were eluted with a second 100 μ l elution buffer and the eluate combined. After adding 30 μ l sodium acetate (pH 5.4), 750 μ l of ethanol was added and the mixture cooled to -75°C for 15 min. The tube was then centrifuged at 14,000 rpm, 4°C for 10 min in an eppendorf 5402 centrifuge. The pellet was then washed with 70% ethanol. After vortexing briefly, the tube was centrifuged at 14000 rpm, 4°C for 10 min. The pellet was then dried in a vacuum chamber for 15 min, desolved in 100 μ l deionized and distilled water and stored at 4°C. The specific activity of the labeled probes were measured using a Beckman LS6000TA liquid scintillation system and were between 12,000 to 15,000 cpm/ μ l.

PCR amplification and labeling of the 49-bp probe

A 49 bp DNA fragment containing a GRE site was generated and labeled with [α - 32 P]dATP using PCR. The primers designed for the PCR reaction were synthesized by the GeneLab at the School of Veterinary Medicine Louisiana State University and are shown in Table 4. The PCR

reaction was carried out in a 50 μ l solution containing 90 ng of EcoRI digested pME925 DNA as template, 1 X PCR buffer, 0.5 mM dTTP, 0.5 mM dGTP, 0.5 mM dCTP, 1.65 μ M (100 μ Ci) [α -³²P]dATP, 1 μ M of each of the primers (ME49A and ME49B), and 1 unit Vent_R[®] (exo⁻) DNA polymerase which does not degrade the PCR product when there are unequal dNTP concentrations and has a fidelity level twice that of Taq DNA polymerase. The thermocycles are described in Table 5. The PCR reaction was then loaded onto a 5% non-denaturing polyacrylamide gel and purified as described above.

Animals

Zucker lean and obese rats from two sources have been used in the described experiments. In the gel retardation experiments obese (*fa/fa*) and lean (*Fa/fa* or *Fa/Fa*) rats were bred from heterozygote parents. For SDS PAGE and 2-D PAGE analysis, we used rats from a Zucker X Brown Norway cross colony to identify known homozygous lean (*Fa/Fa*) and obese (*fa/fa*) rats. This cross provides a restriction fragment length polymorphism close to the *fa* gene that allows determination of the number of *fa* genes carried with an error of less than 1% (257).

All rats were bred in the AALAC-accredited animal facility of the Pennington Biomedical Research Center. They were housed singly, after weaning at 21 days, in hanging wire cages with an automated water system in rooms maintained at 22-24°C with a 12 hour (08.00 to 20.00 hour) light-dark cycle. Food (laboratory rodent #5001, PMI Feeds Inc.) was available *ad libitum*.

Bilateral adrenalectomies or sham-operations were performed under metofane anesthesia as described previously (116). During the immediate 24-hour postoperative period all rats were maintained at 24-26°C, before being returned to normal housing temperature. Adrenalectomized rats were maintained on a 0.9% (w/v) saline drinking solution. Some adrenalectomized rats were treated with dexamethasone (0.5 mg/kg body weight, daily) for two days before sacrifice, which was seven days after the operation. The dexamethasone was injected intraperitoneally in an Tween-20/ethanol/0.9% (w/v) NaCl (1:25:975, by volume) vehicle. All control animals received a similar volume of vehicle. Immediately after decapitation of the rats, trunk blood was collected for the preparation of serum. The success of the adrenalectomy procedure was confirmed by demonstration of undetectable serum corticosterone levels in a radioimmunoassay procedure as previously described (116).

Preparation of hepatic nuclear extracts

Nuclear proteins were prepared from extracts of purified nuclei of lean and obese Zucker rats by the method of Bernal et al. (258). All manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 0°C. When included, DTT, PMSF, Pepstatin A, and Leupeptin were added to the buffers just prior to use. Rats were decapitated at the age of 4-6 weeks. 7.5 g liver was minced and homogenized in SMDP solution containing 0.32 M sucrose, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 1 μM pepstatin A, and 1 μM leupeptin, and centrifuged at 1090 x g in a Beckman J2-21M centrifuge for 10 min. The pellet was washed with SMDP solution, resedimented and resuspended in 2.4 M sucrose containing 1 mM PMSF

and 1 mM DTT. It was centrifuged in a Beckman fixed angle Ti 50.2 rotor at 130,000 xg for 45 min. The pellet was resuspended in 9 ml of SMTDP solution (0.32 M sucrose, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF) to which 250 µl of 10% triton X-100 was added. After incubation at 4°C for 5 min, the pellet was sedimented at 1090 xg in a Beckman J2-21M centrifuge for 10 min and washed with 10 ml of SMTDP solution. The pellet was used as purified nuclear fraction. The purified nuclei were lysed in 10 mM Tris-HCl buffer pH 8.0 containing 0.4 M KCl, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, incubated for 1 hour with occasional mixing, centrifuged in Beckman Ti 50.2 rotor at 150,000 x g for 1 hour. The supernatant was frozen with liquid nitrogen in small aliquots and was stored at -80°C. It was used as nuclear protein extract in the gel retardation studies, SDS PAGE, and 2-D PAGE. Protein content was assayed using the Bio-Rad dye binding method of Bradford (259). Once thawed, the extracts were not refrozen.

Gel retardation studies

Binding reactions were carried out basically as described by Petty et al. (248). 1 or 2 µg of Zucker rat liver nuclear extract was added to 20 µl of binding buffer containing 125 mM Tris-HCl (pH 8.0), 25 mM MgCl₂ (to adjust ionic strength), 2.5 mM EDTA (as a chelation agent), 50% glycerol (to stabilize the binding), 0.1% Nonidet P-40 (to minimize aggregation), 2.5 mM dithiothreitol (as a reducing agent), 250 mM NaCl (to adjust ionic strength), and 0.5 mg/ml poly(dI-dC)·poly(dI-dC) (as a nonspecific competitor DNA). 2 µl of labeled probe was then added and incubation was continued for 20 min at room temperature. After addition of 2 µl of 10X loading buffer (0.1% bromphenol blue and 50% glycerol) reaction mixtures were loaded onto a

1.5 mm x 14 cm x 16 cm polyacrylamide gel (4 or 5%, acrylamide:bisacrylamide, 37.5:1) that had been pre-electrophoresed at 100 V for at least 30 minutes in a buffer of 50 mM Tris, 0.38 M glycine, 2 mM EDTA, pH 8.3. Electrophoresis was carried out at 200 V, 4°C until the bromphenol blue dye front reached the bottom of the gel. Gels were dried and exposed to a Molecular Dynamics Phosphorimager screen or an X-ray film.

Gel shift interference experiments were carried out under the same conditions as described above for the gel retardation assay except that after adding nuclear extract in the binding buffer, the incubations were carried out in the presence of specified amount of phosphatase, protein kinases, or antibodies. CIAP, PKA, or PKC was incubated with the nuclear proteins at 30°C for 30 min. BuGR2 or HNF3 α antiserum was incubated with the nuclear proteins at room temperature for 20 min.

SDS PAGE of proteins in DNA-protein complexes from band shift assays

In order to analyze the proteins present in the bands of interest in the band shift assays, 15 fold scale-up band shift assays were performed with the 222 bp DNA fragment as labeled probe. Band shift assays were carried out as described above except that two control reactions were run without any target DNA added. After exposure to the X-ray films, the original gels were aligned with the X-ray films and the bands of interest were excised from the gel. The gels in which control reactions were loaded were also aligned with the X-ray films obtained from band shift assays performed with labeled probes and the gel slices at the same position were excised. The excised gel slices were chopped into small pieces and the proteins in the gels were

eluted using a Hoefer GE 200 Eluter at 4°C, 200 V for 3 hours. The eluted proteins were then concentrated using an Amicon centricon (10,000 M.W. cut-off) as described above. After denaturation at 95°C for 5 min in the SDS/sample buffer (5% SDS, 5% beta-mercaptoethanol, 10% glycerol and 60 mM Tris, pH 6.8), the concentrated proteins were loaded onto a Millipore pre-cast 4-20% SDS polyacrylamide gel (8 cm x 7 cm x 1 mm) and run at 200 V for 1 hour in a Hoefer SE 250 Mighty Small II Vertical Slab Unit. BioRad SDS-PAGE Molecular Weight Standards was also loaded on the gel to show the sizes of the proteins. The gel was then stained with the BioRad silver stain kit following the instruction provided by the manufacturer. Black and white pictures of the stained gel were then taken using a Nikon 700 camera.

Analysis of the hepatic nuclear proteins using polyacrylamide gel electrophoresis

SDS PAGE

Hepatic nuclear proteins from obese and lean Zucker rats were subjected to SDS PAGE. 5 µg of each of the nuclear extracts was heated at 95°C for 5 min in 50 µl of SDS/sample buffer and loaded onto an 8% SDS PAGE (1.5 mm x 16 cm x 12 cm) . The gel was run at 10 mA until the bromphenol blue dye front reached the bottom of the gel. The gel was then silver stained and its pictures taken as described above.

2-D PAGE

Two-dimensional electrophoresis was performed according to the method of O'Farrell (260) by Kendrick Labs, Inc. (Madison, WI) as follows:

Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm, using 2.0 Resolytes pH 4-8 ampholines (BDH from Hoefer Scientific

Instruments) for 9600 volt-hours. One μg of an IEF internal standard, tropomyosin protein, with lower spot of M.W. 33,000 and pI 5.2 was added to the samples. This standard is indicated by an arrow on the stained 2-D gel.

After equilibration for 10 min in a buffer containing 10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris, pH 6.8, the tube gels were sealed to the top of stacking gels which are on top of 10% acrylamide slab gels (0.75 mm thick) and SDS slab gel electrophoresis carried out for about 4 hours at 12.5 mA/gel. The following proteins were added as M.W. standards to the agarose which sealed the tube gel to the slab gel: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). These standards appear as horizontal lines on the Coomassie Brilliant Blue R-250 stained 10% acrylamide slab gels. The Coomassie blue stained gels were dried between sheets of cellophane with the acid edge to the left.

For protein purification, the slab gels were transferred to transfer buffer (12.5 mM Tris, pH 8.8, 86 mM glycine, 10% methanol) transblotted onto PVDF paper overnight at 4°C, 200 mA and approximately 100 V/2 gels. The membranes were stained in 50% methanol and 0.1% Coomassie Brilliant Blue for 5 min, destained in 50% methanol for 2 min and rinsed 10 minutes six times in ultrapure water. The membranes were then dried between sheets of filter paper. The protein spots of interest were cut out of the PVDF paper with a razor blade and sent to Baylor College of Medicine for gas phase sequencing analysis.

RESULTS

Subcloning of ME 5' flanking region

The 942 bp EcoRI-PstI fragment containing the 5' flanking region was subcloned from pME882-CAT into pUC19 (Figure 5). The entire sequence of the recombinant plasmid was determined using the Wisconsin Sequence Analysis PackageTM (Genetics Computer Group, Inc.). The insert was confirmed by restriction analysis (Figure 3). The sequences of the PCR subcloned inserts in the recombinant plasmids pME222 and pME186 were also determined. The digestion of pME925 with different restriction enzymes gave rise to a 385 bp fragment (AflII-NotI) and a 134 bp fragment (NotI-BamHI) (Figure 4). The digestion of pME222 with EcoRI yielded a 222 bp fragment (Figure 7), while a double digestion of the same plasmid with EcoRI and PflMI generated two other fragments with the length of 70 bp and 152 bp respectively (Figure 8). The 186 bp fragment was obtained by cutting the pME186 with EcoRI (Figure 7).

The TRE, IRE-II, and AP-1 sites fall into the 385 bp fragment. The GRE, IRE-I, and HNF3 sites fall into the 222 bp fragment, with the GRE in the 152 bp fragment, while the IRE-I and HNF3 sites are contained in the 70 bp fragment (Figure 1, Table 2).

Binding of the nuclear extracts to the DNA fragments

The first goal of this study was to verify the hypothesis that the higher hepatic ME mRNA level in obese Zucker rats is caused by enhanced transcription activation of the ME gene. As would be described below, this hypothesis was proven true. The next step was to identify the location of the

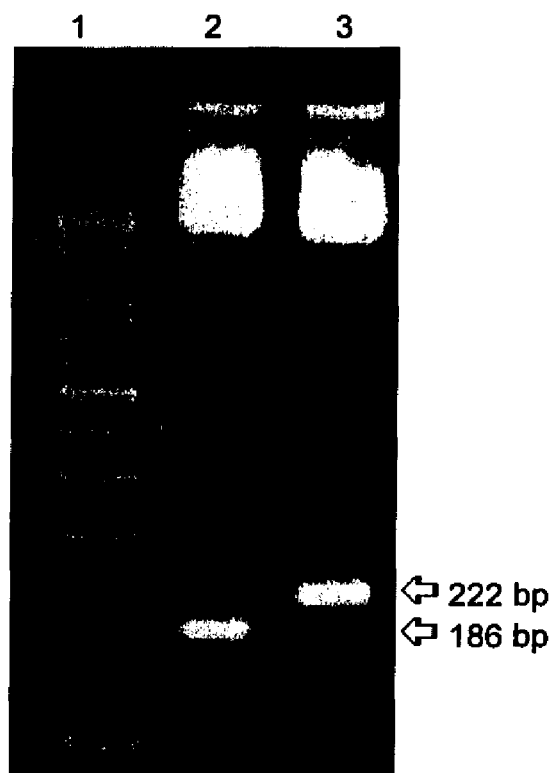


Figure 7. Generation of the 222 bp and 186 bp DNA fragments to be used in the band shift assays. Digestions of the plasmids pME222 and pME186 with EcoRI gave rise to a 222 bp and 186 bp DNA fragments, respectively. Lane 1 shows the 100 bp DNA ladder used as a DNA size marker. Lane 2 (pME186) and 3 (pME222) contain the digestion reactions resolved on a 5% PAGE. Positions of the 222 bp and 186 bp DNA fragments are indicated by the arrows on the right.



Figure 8. Generation of the 152 bp and 70 bp DNA fragments to be used in the band shift assays. Digestions of the plasmids pME222 with EcoRI-PfIMI gave rise to a 152 bp and 70 bp DNA fragments, respectively. Lane 1 shows the 100 bp DNA ladder used as a DNA size marker. Lane 2 contains the digestion reaction resolved on a 5% PAGE. Positions of the 152 bp and 70 bp DNA fragments are indicated by the arrows on the right.

DNA sequences that are bound differentially by the nuclear extracts from obese and lean Zucker rats. Thus the band shift experiments were initially performed with five DNA fragments, which together cover the entire 882 bp upstream region of the ME gene. The images obtained by scanning the phosphorimager screen are shown in Figures 9-10, 12-14.

Band shift assay with the 134 bp DNA

The 134 bp DNA fragment used in the band shift assay shown in Figure 9 was generated by digestion of the plasmid pME925 with NotI and BamHI and is located at -102 to + 33 of the ME gene (Figure 1). Up to seven bands were seen when this 134 bp DNA fragment, which was labeled with [α - 32 P]dATP, was incubated with hepatic nuclear extract from lean and obese rats (Lanes 2 and 3 of Figure 9). The single band in lane 1 showed the position of the free labeled 134 bp DNA.

The specificity of the binding was demonstrated by competition experiments, in which the binding reaction was performed in the presence of excess unlabeled competitor. In lanes 4 and 5, 30-fold of unlabeled target DNA (the 134 bp fragment) was included in the binding reaction as a competitor. As can be seen, the bands that represent the formation of the DNA-protein complexes disappeared with the addition of the unlabeled competitor. Thirty fold of sonicated *E. coli* DNA was included in the binding reactions shown in lanes 6 and 7, and the formation of the DNA-protein complexes were not competed by this unrelated DNA.

There was no significant difference between the levels at which this 134 bp DNA fragment was bound by the hepatic nuclear proteins from obese and lean rats (Appendix A). The overall patterns by which the nuclear

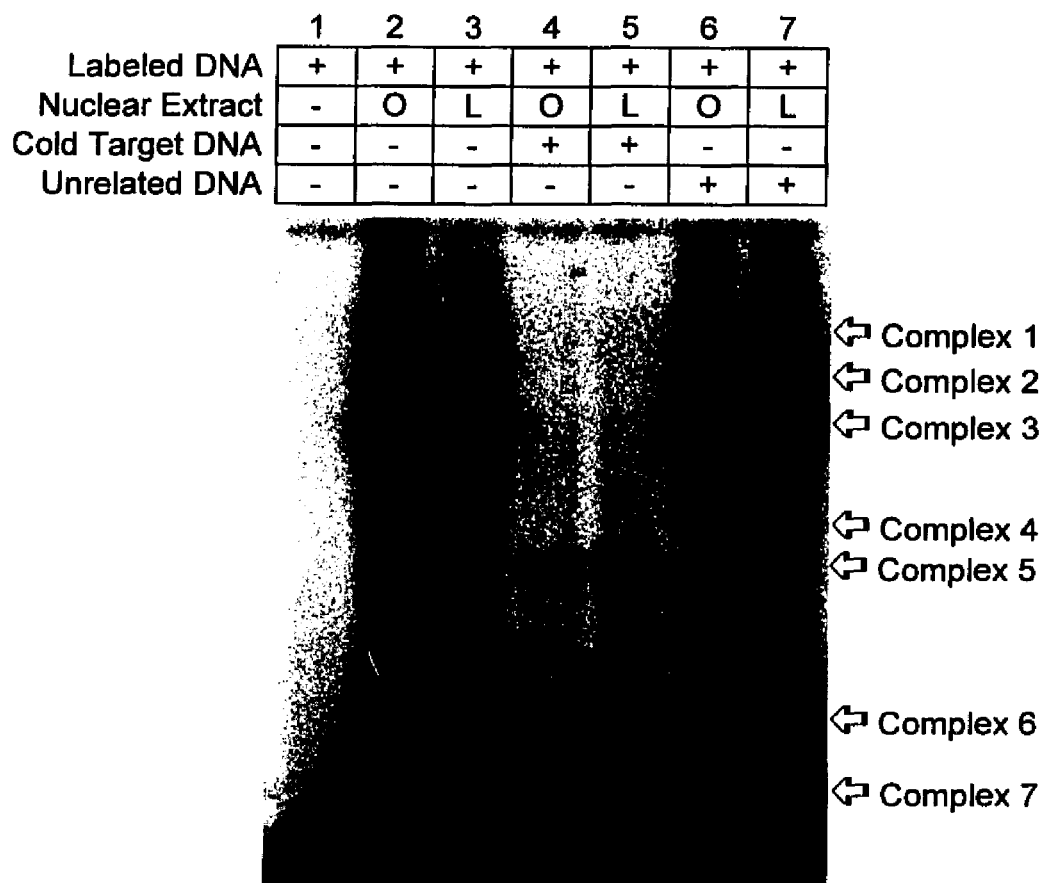


Figure 9. Band shift assay with the 134 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -102 bp to +33 bp region of ME gene, together with or without unlabeled target DNA or E. coli DNA. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

proteins bind to the 134 bp DNA fragment were same in obese and lean animals.

Band shift assay with the 385 bp DNA

The 385 bp DNA fragment located between -488 and -103, the region which contains a TRE, Sp1, AP-1 and the IRE-II. It was prepared by digestion of pME925 with NotI and AflII (Figure 4) and labeled with [α - 32 P]dGTP using Klenow fragment. The binding of the nuclear proteins from obese and lean Zucker rat liver to the labeled 385 bp DNA fragment resulted in four major bands in the band shift assay shown in Lanes 2 (lean nuclear proteins) and 3 (obese nuclear proteins) in Figure 10. The position of the unbound labeled target DNA was shown in lane 1 which serves as a control.

It was obvious in all the four bands shown in lanes 2-3 that more hepatic nuclear proteins from obese Zucker rats bound to the 385 bp DNA when compared to lean Zucker rats (Appendix B). The formation of all the four DNA-protein complexes are specific because the addition of 30-fold excess unlabeled target DNA competed with and replaced the labeled DNA (lanes 4-5) in the bands that were shown in lanes 2-3 and the inclusion of unrelated DNA did not change the formation of any of the bands (lanes 6-7).

The difference between the binding of nuclear proteins from obese Zucker rats and that from lean Zucker rats was in the amount of the DNA that was bound.

Band shift assay with the 222 bp DNA

The region from -712 to -491 of ME gene is covered by a 222 bp DNA fragment which contains an HNF3 binding site, an IRE-I, a TATA box, and a

	1	2	3	4	5	6	7
Labeled DNA	+	+	+	+	+	+	+
Nuclear Extract	-	L	O	L	O	L	O
Cold Target DNA	-	-	-	+	+	-	-
Unrelated DNA	-	-	-	-	-	+	+

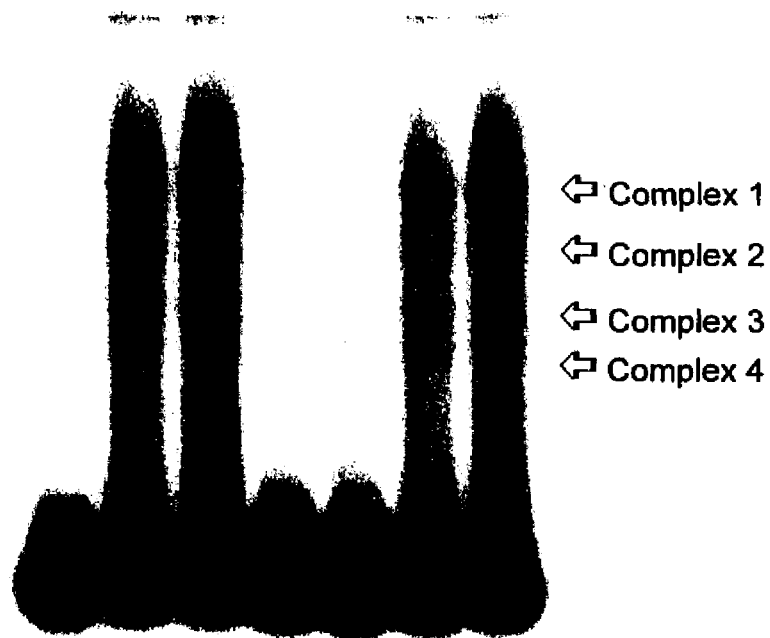


Figure 10. Band shift assay with the 385 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -488 bp to -103 bp region of ME gene, together with or without unlabeled target DNA or E. coli DNA. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

potential GRE site (Figure 1). It was obtained by digestion of pME222 with EcoRI and radioisotope-labeled using Klenow fragment.

At least four bands were seen when this fragment, labeled with [α - 32 P]dATP, was incubated with the nuclear proteins from both obese and lean Zucker rat liver (lanes 2-3 in Figure 11). Lane 1 in Figure 11 has only the labeled 222 bp fragment and was used as a control reaction. Band shift assays performed in the presence of 30-fold of unlabeled 222 bp fragment DNA (lane 4) showed a disruption of the binding by the nuclear proteins to the labeled target DNA. Addition of 30-fold of sonicated E. coli DNA to the band shift assay failed to affect the binding of the nuclear proteins to the labeled target DNA.

Interestingly, the pattern of binding of hepatic nuclear proteins to this 222 bp DNA fragments were clearly different between obese and lean rats (lanes 2-3 in Figure 11). There was more Complex 1 formed by obese nuclear extract than by lean nuclear extract. On the other hand, there was more of complex 2, 3, and 4 formed by lean nuclear extract than by obese nuclear extract (Appendix C). Therefore, this region was selected for more thorough examination as will be described below.

Band shift assay with the 186 bp DNA

The 186 bp DNA fragment from -881 to -696 of ME gene was obtained by digestion of pME186 with EcoRI and labeled with [α - 32 P]dATP using Klenow fragment. It does not contain any known protein binding site. However, there were still two DNA-protein complexes formed when this 186 bp fragment was used in band shift assay (Figure 12). The formation of these DNA-protein complexes was specific because the addition of 30-fold

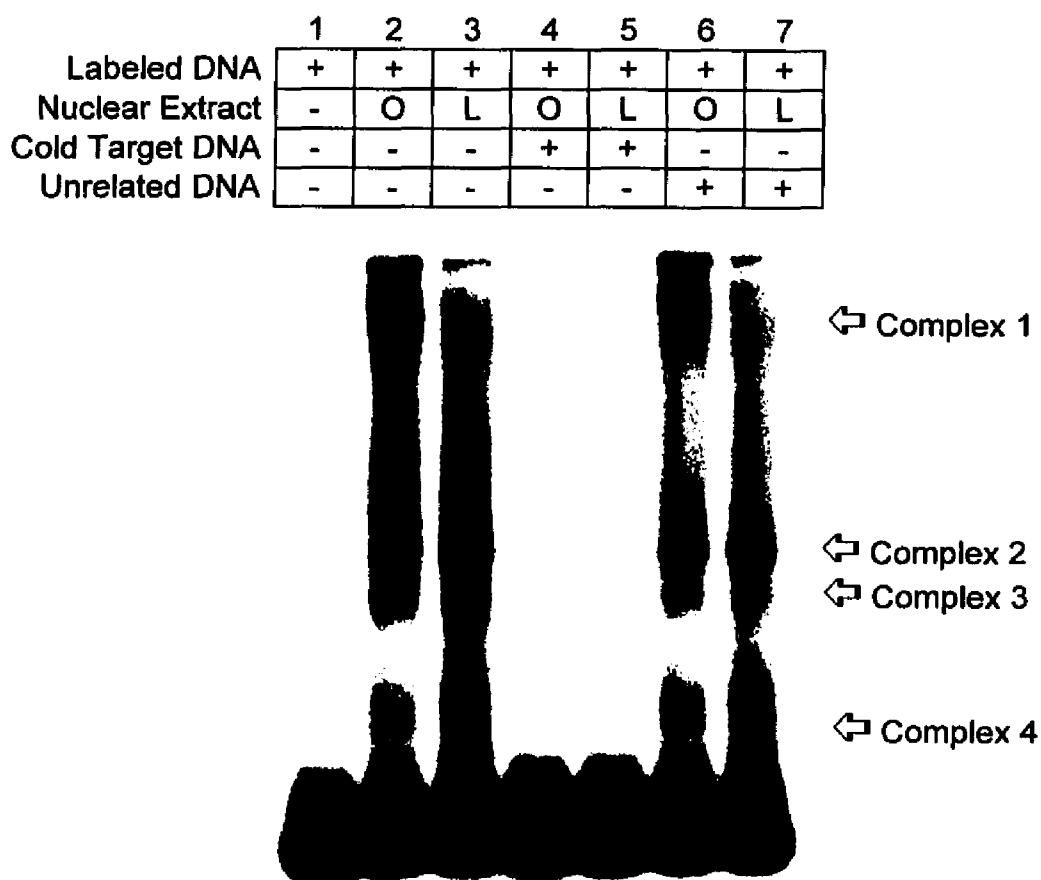


Figure 11. Band shift assay with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene, together with or without unlabeled target DNA or E. coli DNA. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

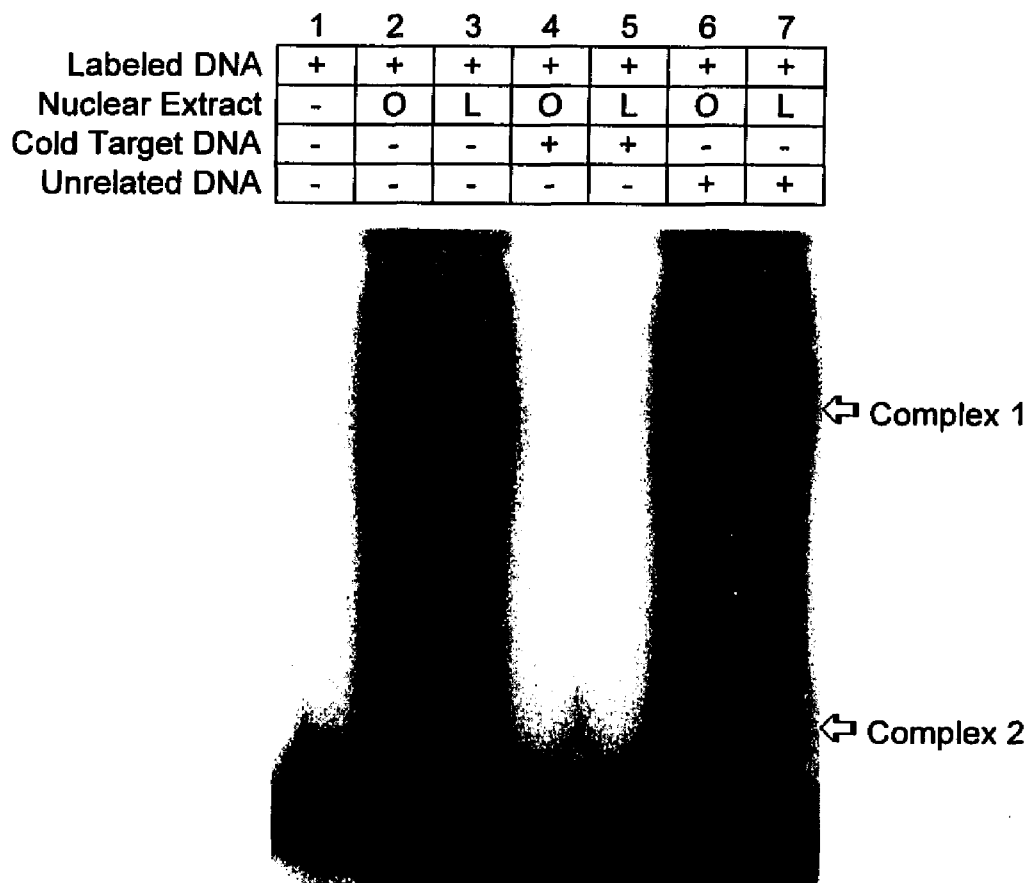


Figure 12. Band shift assay with the 186 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -881 bp to -696 bp region of ME gene, together with or without unlabeled target DNA or E. coli DNA. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

excess of unlabeled 186 bp target DNA negated the formation of the complexes by the nuclear proteins from obese and lean Zucker rat liver, and labeled target DNA and the inclusion of 30-fold excess of unrelated DNA did not affect the formation of these DNA-protein complexes.

There was a difference between the levels at which this 186 bp DNA fragment was bound by the hepatic nuclear proteins from obese and lean rats. It was bound at a much higher level by nuclear proteins from obese rat liver than by the ones from lean rat (Appendix D). However the pattern of binding of hepatic nuclear proteins was similar in extracts from obese and lean rats.

Band shift assay with the 49 bp DNA

To investigate the binding of the nuclear proteins from obese and lean Zucker rat liver to the GRE site, a 49 bp DNA fragment was generated and labeled with [α -³²P]dATP using PCR (Figure 13) as described in Materials and Methods. It covers the region -511 to -463 of the ME gene and contains only a GRE site (Figure 1, Table 4).

A single DNA-protein complex was seen when this 49 bp DNA fragment was used as labeled probe in the band shift assay with nuclear proteins from obese and lean Zucker rat liver (lanes 2 and 5 in Figure 14). There was a higher concentration of proteins that bind to this 49 bp DNA fragment in obese Zucker rats when compared to lean (Appendix E). The formation of these DNA-protein complexes was specific because the addition of 30-fold excess unlabeled target DNA competed with and replaced the labeled DNA (lanes 3 and 6) in the bands that were shown in lanes 2 and 5



Figure 13. Generation of the 49 bp DNA fragment to be used in the band shift assays. PCR amplication using the 925 bp DNA fragment as template and the oligonucleotides ME49A and ME49B as primers gave gave rise to a 49 bp and 70 bp DNA fragments, respectively. Lane 1 shows the 100 bp DNA ladder used as a DNA size marker. Lane 2 contains the digestion reactions resolved on a 5% PAGE. Positions of the 49 bp DNA fragments are indicated by the arrow on the right.

	1	2	3	4	5	6	7
Labeled DNA	+	+	+	+	+	+	+
Nuclear Extract	-	O	O	O	L	L	L
Cold Target DNA	-	-	+	-	-	+	-
Unrelated DNA	-	-	-	+	-	-	+

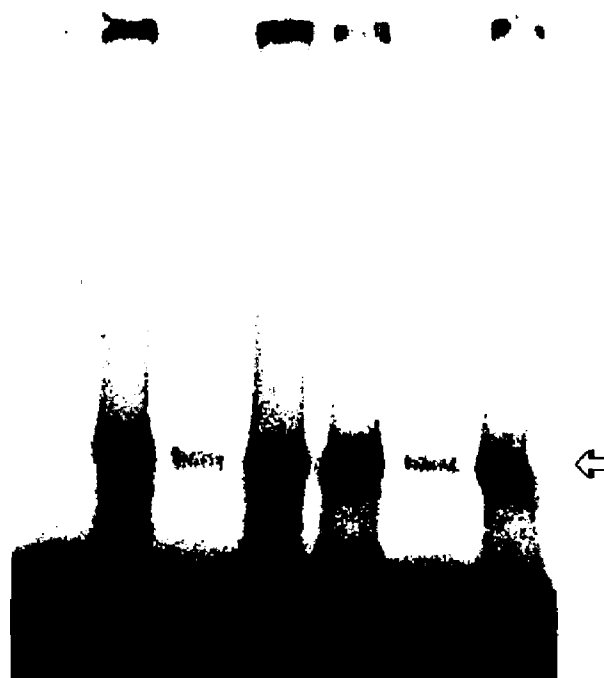


Figure 14. Band shift assay with the 49 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -511 bp to -463 bp region of ME gene, together with or without unlabeled target DNA or E. coli DNA. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrow indicates the position of the shifted complex. +: present, -: absent, O: obese, L: lean

and the inclusion of unrelated DNA did not change the formation of any of the bands (lanes 4 and 7).

Adrenalectomy abolishes the binding of nuclear extract to ME222

Because adrenalectomy has been reported to be able to prevent or suppress the development of almost all the experimental obesity models, including Zucker rats (46), and the mRNA level of hepatic ME in obese Zucker rats has also been shown to be reduced to the level of that in lean Zucker rats (130), it was of interest for us to investigate the effect of adrenalectomy on band shift patterns with the upstream region of the ME gene. Since the pattern of binding of the hepatic nuclear proteins from obese and lean Zucker rats to the 222 bp DNA fragment, which contains a GRE site, an IRE-I site, and an HNF3 binding site, was shown to be different. We chose this 222 bp DNA fragment to further study the effect of the status of adrenal glucocorticoids on the binding of the hepatic nuclear proteins.

When nuclear extracts from sham animals were used in the band shift assay, there was more Complex 1 formed by hepatic nuclear proteins from obese animals than by that from lean animals (lanes 2-3 in Figure 15, Appendix F), and there were slightly less Complexes 2-4 formed by hepatic nuclear proteins from obese Zucker rats than by those from lean Zucker rats. This was consistent with the results obtained from the band shift assays performed with nuclear extracts from obese and lean Zucker rats that did not receive any surgery. This indicated that the sham operation did not affect the binding of the nuclear proteins to the 222 bp fragment. The single band in lane 1 in Figure 15 indicated the position of the labeled 222 bp target DNA when it was not bound by any proteins.

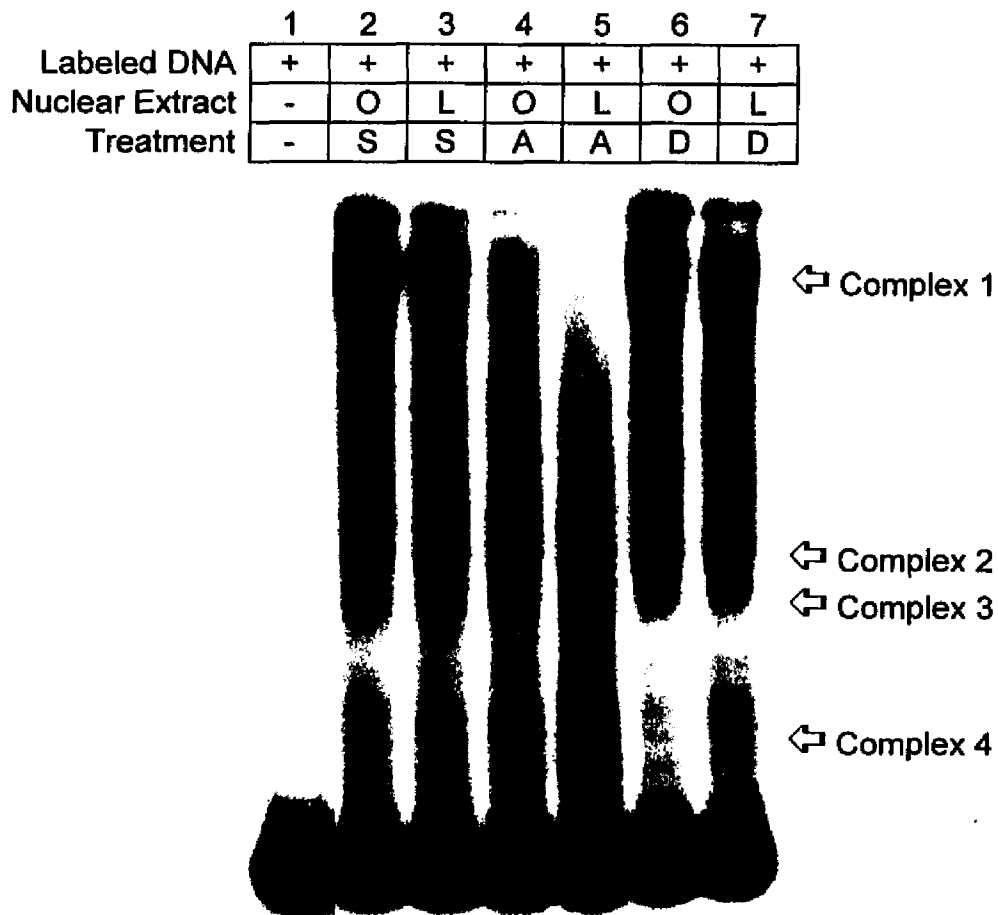


Figure 15. The effect of adrenalectomy on band shift pattern with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been adrenalectomized, adrenalectomized and dexamethasone treated, or sham operated, were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean, S: sham, A: adrenalectomy, D: dexamethasone.

The formation of Complex 1 by adrenalectomized obese rat nuclear extracts was reduced to approximately the level lower than that of sham lean rat nuclear extracts (lane 4) and the Complex 1 formed by adrenalectomized lean nuclear extracts was reduced even further (lane 5). After dexamethasone treatment, the obese adrenalectomized rat nuclear extracts evidenced a larger amount of proteins that bind to the 222 bp DNA fragment than sham obese rat nuclear extracts (lane 6 in Figure 15, Appendix F) and the lean adrenalectomized rat nuclear extracts bound in a higher level to the 222 bp DNA fragment than sham lean rat nuclear extracts although still slightly lower than sham obese nuclear extracts.

The levels of complexes 2-4 formed by the hepatic nuclear proteins from adrenalectomized and dexamethasone replaced obese and lean Zucker rats were affected by the glucocorticoid status manipulation in an opposite way to the levels of Complex 1.

Dephosphorylation of nuclear proteins abolished the binding

Protein phosphorylation is one of the most frequent forms of posttranslational modification in eukaryotic cells and is linked to the control of a variety of cellular functions (158, 222). Glucocorticoid receptor, a steroid hormone receptor, is a phosphoprotein and undergoes hyperphosphorylation after hormone-induced activation (177). Insulin receptor, a representative of peptide hormone receptors, is not only a phosphoprotein, but also one of the receptor tyrosine kinases (184-189). Based on our above observation that there were differences between the patterns with which the hepatic nuclear proteins from obese and lean Zucker rats bind to the 222 bp DNA fragment, which contains both an IRE-I and a GRE, it was rational to further investigate

the influence of the phosphorylation status of the hepatic nuclear proteins on their binding to the 222 bp DNA fragment.

First, we used CIAP to dephosphorylate the hepatic nuclear proteins from obese and lean Zucker rats. 0.2-4 units of CIAP was incubated with the nuclear proteins in the band shift reactions for 30 min before labeled probe was added. As the amount of the CIAP increased, the formation of the specific DNA-protein Complexes 1-3 decreased, regardless of whether the hepatic nuclear proteins used in the band shift assays were from obese Zucker rats (lanes 4, 6, 8, 10, 12 in Figure 16) or lean Zucker rats (lanes 5, 7, 9, 11, 13 in Figure 16, Appendix G). This suggested that one or more of the proteins that formed Complexes 1-3 need to be phosphorylated in order to bind to the DNA or interact with each other.

In contrast, the intensity of Complex 4 was increased when the amount of the CIAP increased, this was the case with complexes formed by both obese and lean Zucker rats.

Phosphorylation of nuclear proteins did not increase the binding

Ideally, phosphorylation of the hepatic nuclear proteins from lean and obese Zucker rats should reverse the effects of dephosphorylation on the formation of the specific DNA-protein complexes. Specifically, it should increase the formation of Complexes 1-3 and decrease the formation of Complex 4. In order to verify if this was the case, PKA, a cAMP-dependent protein Serine/Threonine kinase, was used to phosphorylate the hepatic nuclear proteins from obese and lean Zucker rats. It was added into the binding reactions and incubated for 30 min just before the addition of labeled 222 bp DNA fragment to initiate the binding. But, as indicated in Figure 17,

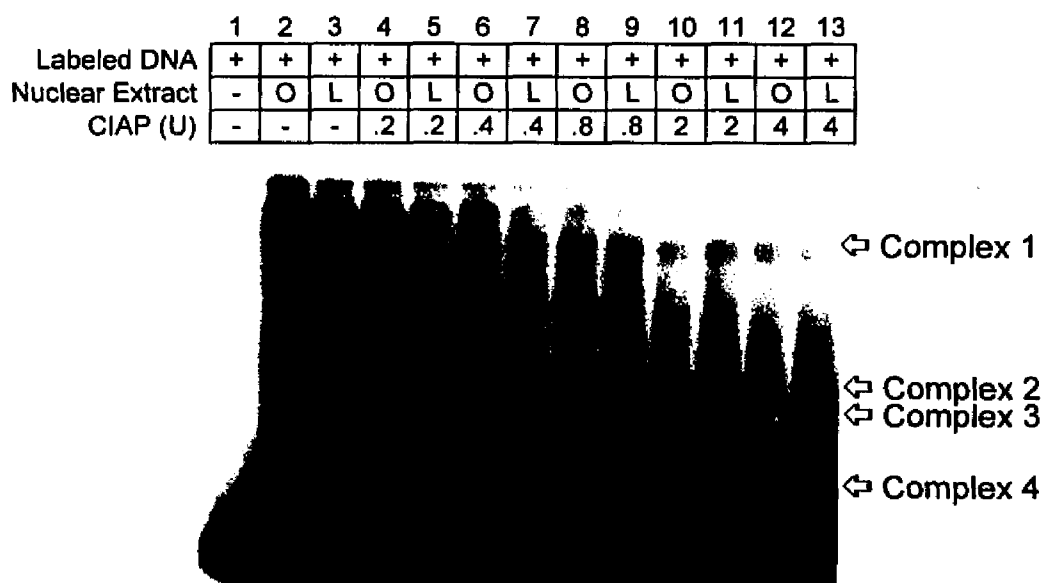


Figure 16. Effect of dephosphorylation on band shift pattern with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been treated with specified amount of CIAP were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

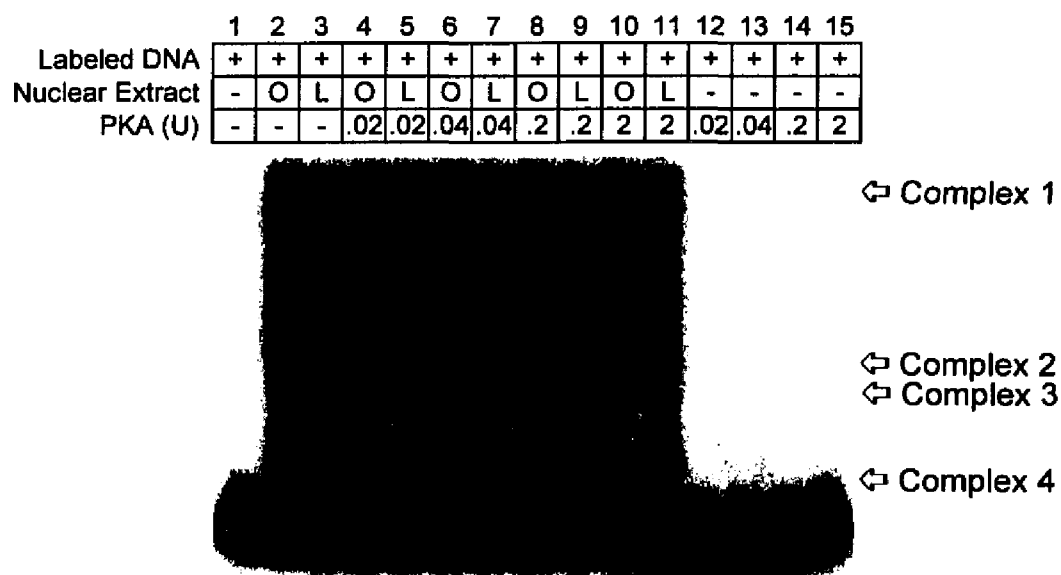


Figure 17. Effect of PKA treatment on band shift pattern with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been treated with specified amount of PKA were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

phosphorylation of the hepatic nuclear proteins with PKA did not change their binding to the 222 bp DNA fragment (lanes 4-11), as compared to the control reactions in which no PKA was used (lanes 2-3). Lanes 12-15 had no nuclear proteins and contained different amount of PKA. No extra bands other than the free probe was detected in lanes 12-15, indicating that PKA, although a protein itself, did not specifically bind to the 222 bp DNA fragment.

Suspecting that PKA was not the appropriate protein kinase to phosphorylate the nuclear proteins involved in the formation of the specific DNA-protein complexes in vivo, we explored the effect of phosphorylation of the hepatic nuclear proteins using PKC, another protein Serine/Threonine kinase which is activated by phosphatidylserine, diacylglycerol, and Ca^{++} . With the addition of PKC in the binding reactions, phosphatidylserine and diacylglycerol, which were incubated with PKC for activation, were also present in the binding reactions. One DNA-protein complex was seen when the PKC treated hepatic nuclear proteins from obese and lean Zucker rats were incubated with the labeled 222 bp DNA fragment. The intensity of the complex was higher when the nuclear proteins from obese animals were used than when those from lean animals were used, which was consistent with the result displayed in complex 1 when no diacylglycerol were included in the binding reaction (Figure 11). However, when PKC was included in the binding reactions, the DNA-protein complexes formed by the binding to the 222 bp DNA fragment by the hepatic nuclear proteins from both obese and lean Zucker rats were dramatically reduced (lanes 4-5 in Figure 18). PKC

	1	2	3	4	5	6
Labeled DNA	+	+	+	+	+	+
Nuclear Extract	-	O	L	O	L	-
PKC (mU)	-	-	-	.3	.3	.3

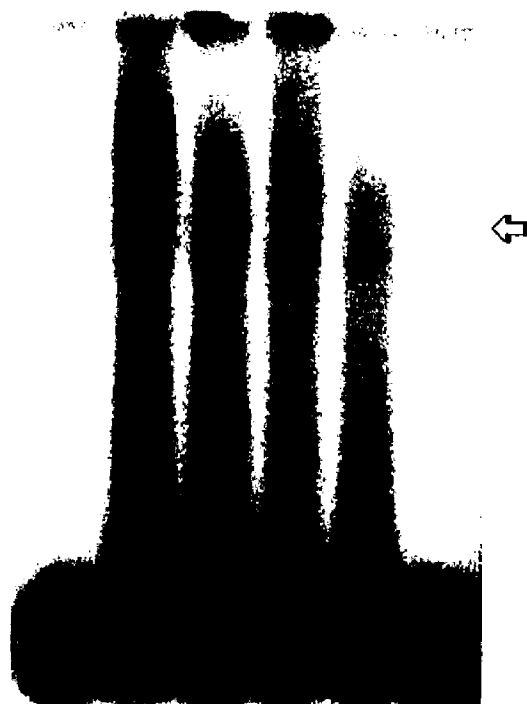


Figure 18. Effect of PKC treatment on band shift assay with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been treated with specified amount of PKC were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrow on the right indicates the position of the shifted complex. +: present, -: absent, O: obese, L: lean.

itself was shown not to bind to the labeled target DNA in lane 6, in which on nuclear proteins were added.

Binding of nuclear proteins to different regions of the 222 bp fragment

In order to further investigate the roles the different regions of the 222 bp DNA fragment played in the formation of the DNA-protein complexes, we obtained two smaller DNA fragments, which together constitute the whole 222 bp upstream region of ME gene (Figure 1), by digesting pME222 with the restriction enzymes EcoRI and PflMI. The resulting 70 bp (containing the HNF3 binding site and the IRE-I site) and 152 bp (containing the GRE site and TATA box) DNA fragments were end labeled with [α - 32 P]dATP using Klenow fragment.

The binding of the hepatic nuclear proteins from both obese and lean Zucker rats to the 70 bp DNA fragment (-712 to -643) resulted in up to six DNA-protein complexes (Figure 19). The formation of the Complexes 1-2 were so weak that they appeared as two smears rather than two bands (lanes 1-2 in Figure 19). In spite of the weak binding, dephosphorylation of the nuclear proteins with CIAP completely abolished the already weak formation of the DNA-protein complex (lanes 4-9, Figure 19). The formation of Complex 3 was also reduced by the dephosphorylation of the nuclear proteins from both obese and lean rats. In contrast, Complexes 5-7 were found increased by the same treatment. Interestingly, another band, named Complex 4, appeared with the dephosphorylation of the nuclear proteins and its intensity increased as the amount of CIAP was used. In all cases, the formation of Complexes 1-3 were stronger in obese animals than in lean animals when same amount of CIAP were used.

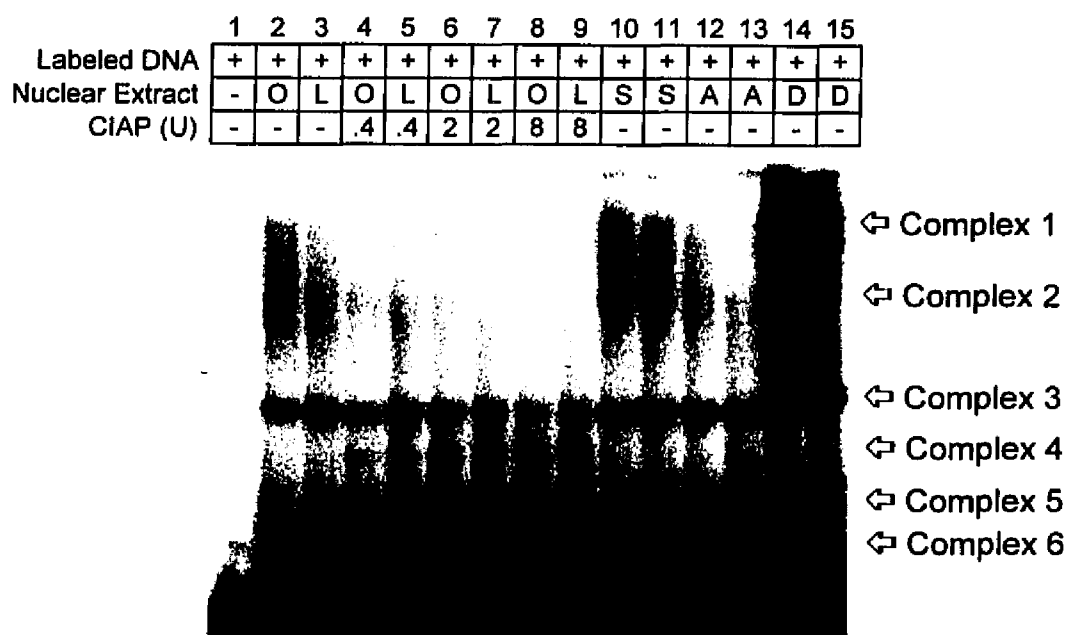


Figure 19. Effect of dephosphorylation and adrenalectomy on band shift pattern with the 70 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been treated with specified amount of CIAP (lanes 2-9) or those from obese and lean Zucker rats that had been adrenalectomized, adrenalectomized and dexamethasone treated, or sham operated (lanes 10-15) were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -643 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean, S: sham, A: adrenalectomy, D: dexamethasone.

The effects of adrenalectomy of the rats on the binding of the nuclear proteins to this 70 bp DNA fragment were also explored. Although there is no GRE site in this region, the formation of the Complexes 1-3 were still inhibited by adrenalectomy of both obese and lean Zucker rats (lanes 12-13 in Figure 19). This treatment had an opposite effect on the formation of Complexes 4-5. Higher amounts of Complexes 1-3 were formed with extracts from obese animals than from lean animals, while the formation of Complexes 5-7 were more prominent in lean animals than in obese animals.

When hepatic nuclear proteins from dexamethasone replaced obese and lean Zucker rats were incubated with the 70 bp DNA fragment, the intensities of Complexes 1-3 were dramatically increased and the formation of complexes 5-7 was greatly reduced compared with those in adrenalectomized rats. Again, the levels of Complexes 1-3 were higher in obese animals than in lean animals and the levels of Complexes 5-7 were higher in lean animals than in obese animals.

When the 152 bp DNA fragment (-642 to -491) was used as the target DNA in band shift assays, up to five bands showed up and there were more nuclear proteins from obese Zucker rats that bound to the 152 bp DNA than from lean Zucker rats (lanes 2-3 in Figure 20). As increasing amounts of CIAP were added in the binding reactions, Complexes 1-2 gradually disappeared and the formation of Complexes 3-5 were increased (lanes 4-9 in Figure 20). When the same amounts of CIAP were added, the formation of Complexes 3-5 were found to be formed in larger amounts in lean animals than in obese animals.

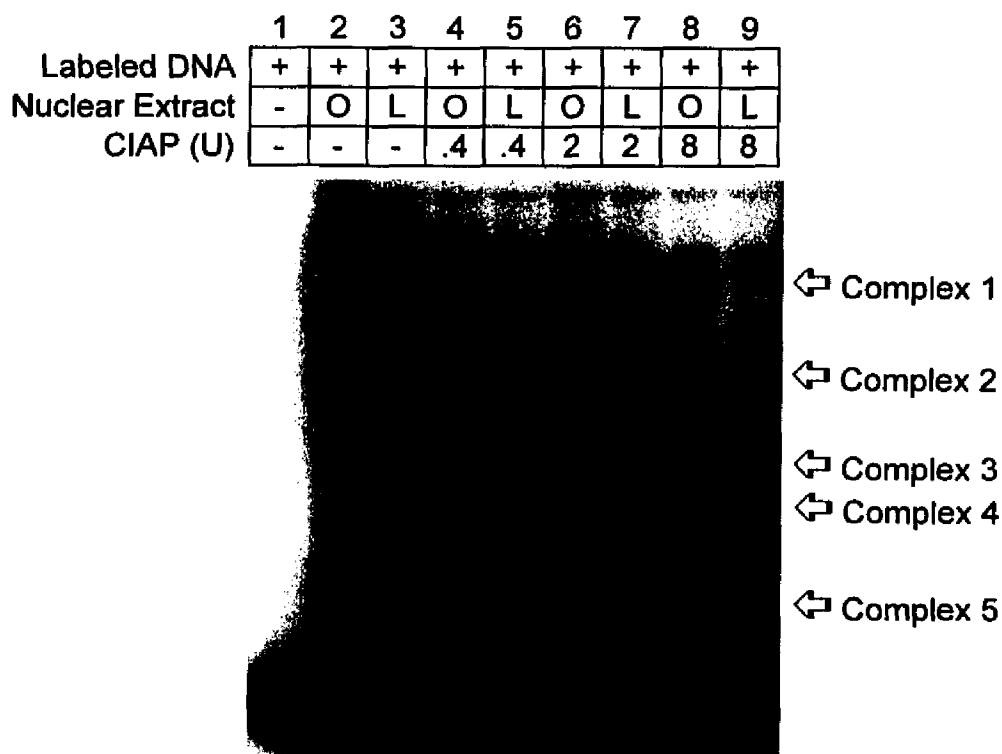


Figure 20. Effect of dephosphorylation on band shift pattern with the 152 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats that had been treated with specified amount of CIAP were incubated with ^{32}P -labeled DNA fragments containing the -642 bp to -491 bp region of ME gene. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

BuGR2 reduced the binding of hepatic nuclear proteins to the 222 bp fragment

Since the glucocorticoid status in obese and lean Zucker rats showed a dramatic influence on the binding of the hepatic nuclear proteins to the 222 bp DNA fragment, which contains a GRE, it was of value to investigate whether the glucocorticoid receptor exists in the DNA-protein complexes formed in the band shift assays. In one of the band shift assays, we treated the hepatic nuclear extracts from obese Zucker rats with 10 units of BuGR2 before the binding reaction took place (lane 3 in Figure 21). Generally the binding of hepatic nuclear proteins to this 222 bp DNA fragment was considerably reduced by the inclusion of the BuGR2 in the binding reaction. This decrease in binding can be observed in the formation of all the DNA-protein complexes. Interestingly, a very faint band showed up at the position slightly below the well. This relatively faint band could not be caused by the binding of the BuGR2 antibody to the labeled 222 bp DNA fragment because incubation of BuGR2 with only the labeled probe showed no shifted band (data not shown).

Lanes 4-6 in Figure 21 shows the effect of different amount of BuGR2 on the formation of the DNA-protein complexes. As the amount of BuGR2 included in the binding reactions was increased (0.1, 1, 2 units), the formation of the DNA-protein complexes decreased except the band just below the well, which might be a super-shifted band.

Therefore, it seems that the glucocorticoid receptor antibody has an inhibitory effect on the binding of the hepatic nuclear proteins from obese Zucker rats to the 222 bp DNA fragment.

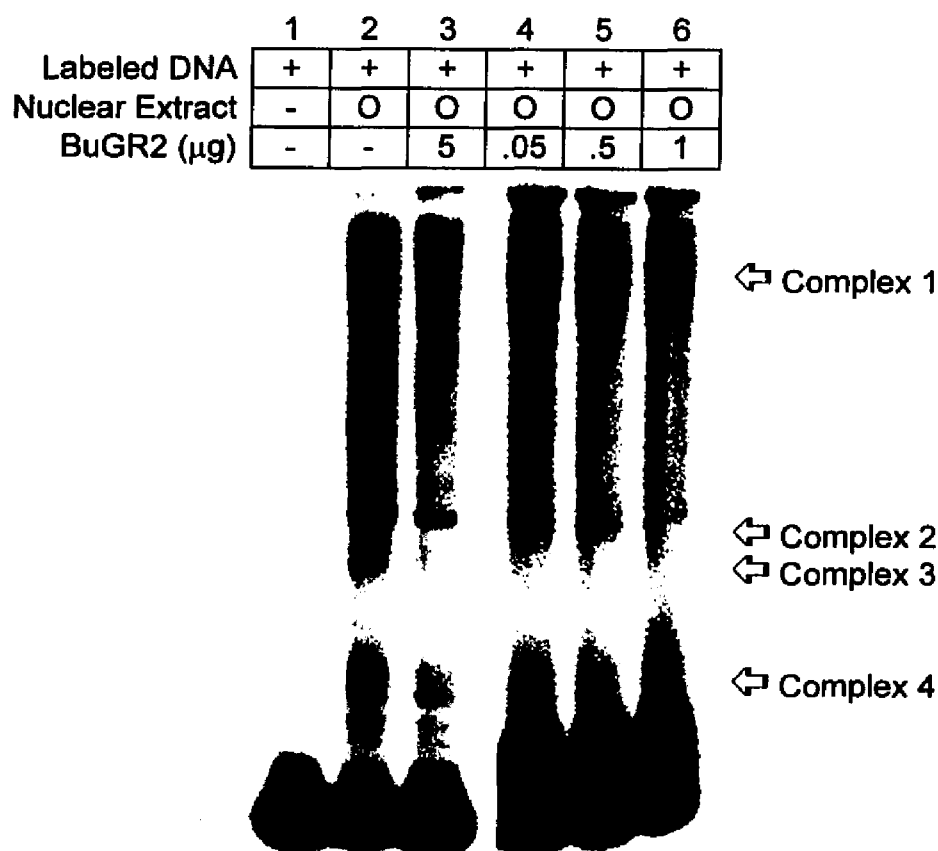


Figure 21. Effect of GR antibody on band shift assay with the 222 bp fragment. Hepatic nuclear extracts prepared from obese Zucker rats were incubated with 32 P-labeled DNA fragments containing the -712 bp to -491 bp region of ME gene, together with specified amount of monoclonal antibodies (BuGR2) against GR. DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

Effects of the anti-HNF3 α antiserum on the binding of hepatic nuclear proteins to the 222 bp DNA fragment

Since the 222 bp DNA fragment also contains an HNF3 binding site and the binding of the hepatic nuclear proteins from obese and lean Zucker rats were significantly affected by the phosphorylation status of the nuclear proteins, we looked into the effect of the treatment of the hepatic nuclear proteins with the anti-HNF3 α antiserum on the formation of the DNA-protein complexes (Figure 22).

Surprisingly, when the anti-HNF3 α antiserum was included in the binding reactions, two extra bands (Complexes a, b) were seen (lanes 4-5 in Figure 22) compared with the band shift assays carried out with only the labeled probe and the hepatic nuclear proteins from obese (lane 2 in Figure 22) and lean (lane 3 in Figure 22) Zucker rats. There was more Complex a formed by obese nuclear extract than by lean nuclear extract. In contrast, there was more of Complex b formed by lean nuclear extract than by obese nuclear extract.

The formation of the four DNA-protein complexes by the binding of nuclear proteins not treated with the anti-HNF3 α antiserum were still observed when the band shift assays were performed with the inclusion of the antiserum. The patterns and intensities of three of these bands (Complexes 1, 2, and 4) were not significantly affected when the nuclear proteins were treated with the antiserum.

The most significant change caused by the treatment with the anti-HNF3 α antiserum was the dramatic increase of the intensity of Complex 3. However, there was still more Complex 3 formed by the obese nuclear

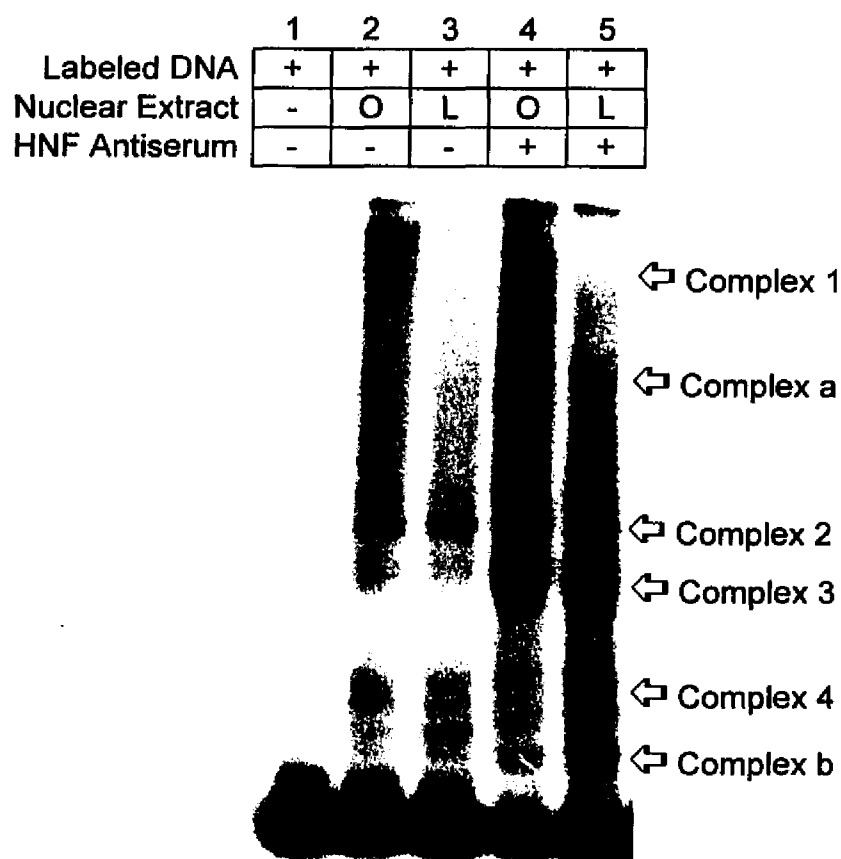


Figure 22. Effect of HNF3 Ab on band shift pattern with the 222 bp fragment. Hepatic nuclear extracts prepared from obese and lean Zucker rats were incubated with ^{32}P -labeled DNA fragments containing the -712 bp to -491 bp region of ME gene, together with or without polyclonal antiserum against HNF3 α . DNA-protein complexes were size fractionated in nondenaturing polyacrylamide gels and were detected by autoradiography. The arrows on the right indicate the positions of the shifted complexes. +: present, -: absent, O: obese, L: lean.

extracts (lane 4 in Figure 22) than that formed by the lean nuclear extracts (lane 5 in Figure 22).

It seems that the anti-HNF3 α antiserum increased the binding of the hepatic nuclear proteins from obese and lean Zucker rats to the 222 bp DNA fragment because there was much less free labeled probe left when the antiserum was used in the binding reaction.

SDS PAGE analysis of proteins in DNA-protein complexes from band shift assays

In order to analyze the proteins present in the Complex 1 in Figure 11, 15 fold scale-up band shift assays were performed with the 222 bp DNA fragment as labeled probe. Two control reactions were run without any target DNA. After exposure to the X-ray films, the original gels were aligned with the X-ray films and the gel slices at the same position as Complex 1 were excised. The proteins in the excised gel slices were eluted and subjected to 4-20% SDS-PAGE analysis. As shown in Figure 23, there were at least 3 proteins in Complex 1 formed by the binding of hepatic nuclear proteins to the 222 bp DNA fragment (lanes 2 and 4). The sizes of the proteins were approximately 123 kd, 67 kd, and 31 kd. There were larger amount of proteins in Complex 1 from obese animal (lane 2) than that from lean animal (lane 4). The protein complex in the unbound nuclear extracts which has the same migration rate as the DNA-protein Complex 1 seems to contain more proteins of smaller sizes than Complex 1 (lanes 1 and 3 in Figure 23). A protein with a M.W. of approximately 33 kd was present in the unbound lean protein complex and absent from the unbound obese protein complex.

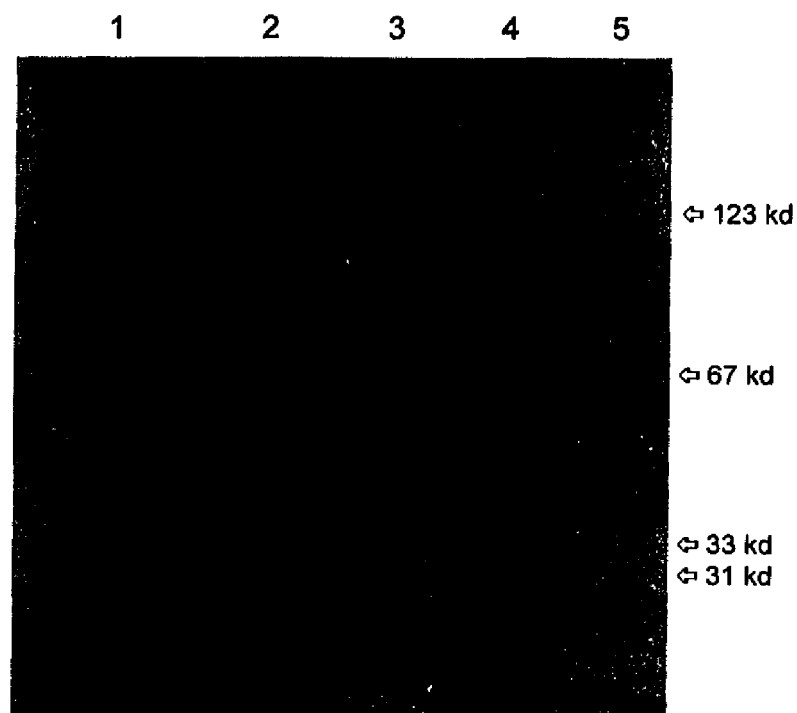


Figure 23. SDS PAGE of Complex 1. The DNA-protein complex 1 indicated in Figure 11 were isolated from a 15 fold scale-up reaction and subjected to SDS-PAGE analysis. The protein complexes from the same position in the band shift assays performed without adding any DNA were also obtained and analyzed. Lane 1, obese nuclear proteins at the same position of Complex 1. Lane 2, obese Complex 1. Lane 3, lean nuclear proteins at the same position of Complex 1. Lane 4, lean Complex 1. Lane 5, protein marker. The protein bands referred in the text were indicated by the arrows.

Analysis of the hepatic nuclear proteins using polyacrylamide gel electrophoresis

Identification of the proteins that are apparently present at higher or lower levels in, or even absent from, the obese rat hepatic nuclei and information of their function and regulation may provide insight into the abnormal responsiveness of certain genes to glucocorticoid in the obese rat.

SDS PAGE

SDS PAGE of the hepatic nuclear proteins from obese and lean Zucker rats showed a large number of bands with a wide range of molecular sizes (Figure 24). No obvious differences were observed in the gel patterns of the obese (lane 2) and lean (lane 3) hepatic nuclear extracts. They both have the same number of bands with approximately identical intensities.

2-D PAGE

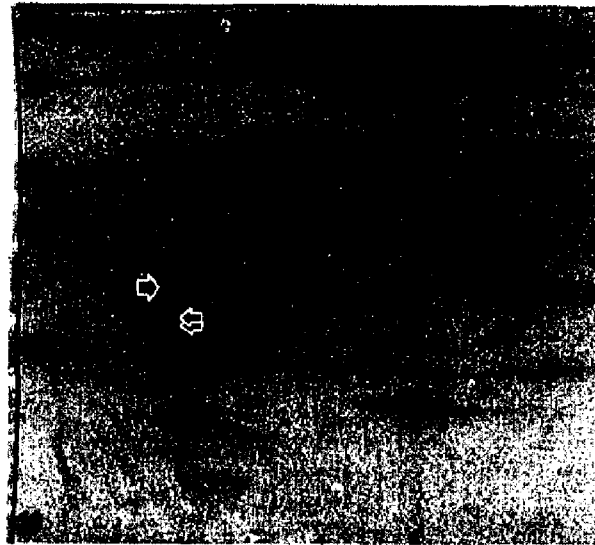
Since no obvious differences were detected between the nuclear protein profiles of obese and lean Zucker rats using SDS-PAGE, the nuclear proteins were subjected to 2-D PAGE, which has a much higher resolving power in detecting individual proteins or polypeptides.

2-D PAGE analysis of the hepatic nuclear extracts from obese and lean Zucker rats showed that the overall composition of nuclear protein profiles were generally similar (Figure 25) except that there were two extra spots in lean nuclear extract which were absent from the obese nuclear extract (Figure 25-26). They have the same pI which is 5.2. Their M.W. were approximately 31 kd and 32 kd, respectively. Attempting to characterize these proteins, the protein spots which had been transferred from the gels to the PVDF membranes were isolated for sequencing analysis. Unfortunately,



Figure 24. SDS PAGE of nuclear proteins from obese and lean Zucker Rats. Hepatic nuclear extracts prepared from obese and lean Zucker rats were analyzed using an 8% SDS-PAGE. Lane 1, lean nuclear extract. Lane 2, obese nuclear extract. Lane 3, protein marker.

A. Lean



B. Obese

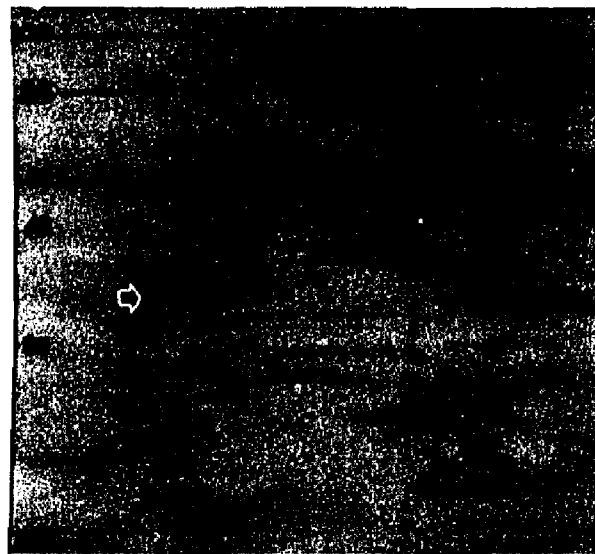
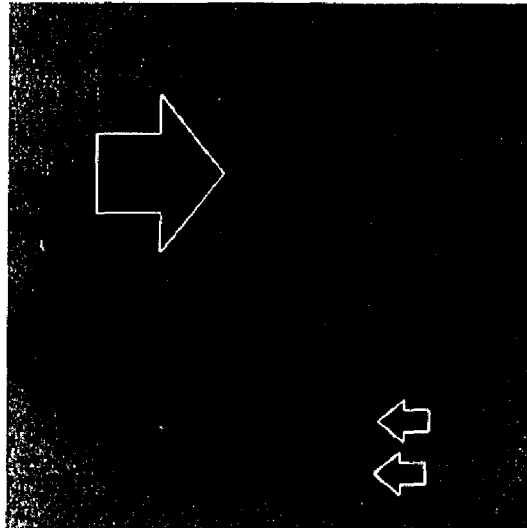


Figure 25. 2-D PAGE of hepatic nuclear proteins from lean and obese Zucker rats. A, lean nuclear extract; B, obese nuclear extract. The 2-D PAGE were performed as described in the Materials and Methods. The two spots (31 and 32 kd respectively) in lean nuclear extract are pointed by the left arrows. The right arrows indicate the position of the internal marker tropomyosin.

A. Lean



B. Obese

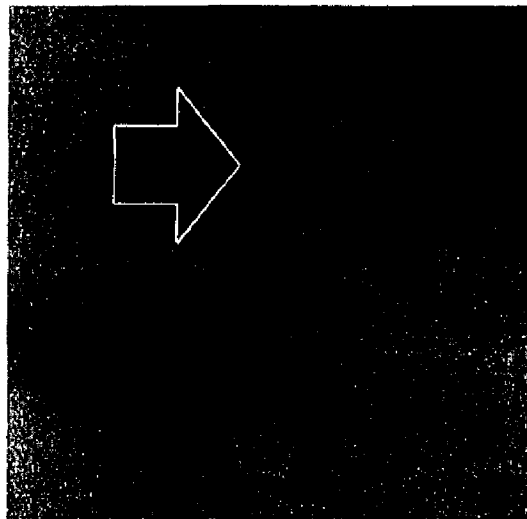


Figure 26. A close up look at the 2-D PAGE of hepatic nuclear proteins from lean and obese Zucker rats. Legends are the same as in Figure 25.

the N-terminals of these two proteins were blocked and the sequences of them could not be obtained.

DISCUSSION

Our initial band shift assays performed with five DNA fragments which cover the entire 882-bp upstream region of the ME gene showed that generally larger amounts of specific DNA-protein complexes were formed when hepatic nuclear proteins from obese rats were used in comparison to hepatic nuclear proteins from lean rats (Figures 9-10, 12-14).

Bray and York (118) have previously shown that the hepatic and adipose mRNA levels for ME and GAPDH were increased in young obese *fa/fa* rats. Our results from the band shift assays indicated alteration in binding of proteins to the upstream region of ME gene. These may contribute to the increase of ME transcription shown by Jensen et al. (261).

The upstream region of the rat ME gene has many known binding sites (Figure 1) for transcription factors, many of which have been well studied (Table 2). These protein binding sites have been found to exist in many eukaryotic genes that are under the control of multiple hormones (262).

The binding of hepatic nuclear proteins to the 134 bp fragment

Up to seven DNA-protein complexes were detected in the band shift assay using the 134 bp DNA fragment as target DNA and hepatic nuclear extracts from obese and lean Zucker rats as proteins (Figure 9). This is not surprising because the DNA sequences in the 134 bp fragment used in band shift assays is located around the cap site (-102 to +33) and have been shown to be bound by many hepatic nuclear proteins (249). The function of this region seems to account for the basal expression of the ME gene, which

has been suggested to be one of the constitutive or "house keeping" genes (263).

Morioka et al. have found a 10-bp direct repeat of the sequence 5'-CTCGCCACCC-3' between -73 and -51, separated by 3 bp. Using DNase I footprint analysis Petty et al. have been able to demonstrate that each copy of the repeated sequence was bound by a separate set of proteins (249). Deletion mutation experiments and transcription competition analysis also showed that this direct repeat is important to maintain the basal transcriptional activity of the ME gene (249). Although the function of this rarely seen direct repeat is still not clearly understood, it has been shown to be bound by rat hepatic nuclear proteins in a thyroidal status dependent manner (249). It is possible that the nuclear proteins which bind to this direct repeat are required for the assembly of the specific transcription initiation complex for the ME gene.

Three of the nine CCGCCC hexanucleotide boxes possessed by the ME gene, which have been speculated to be involved in the chromatin assembly required for gene expression, were also found to be in this 134 sequence (245). Moreover, it has been reported that the sequences between -29 and +28 in this region were protected from DNase I in the footprinting experiment and were essential for the basal transcription activity, indicating that it might be bound by the transcription initiation complex or some of its components.

Therefore the seven bands observed in the band shift assay performed with the 134 bp DNA fragment might represent DNA-protein complexes formed by several different transcription factors or by different

combinations of several transcription factors involved in the transcription initiation.

Band shift assays performed by other researchers showed that most of the sequences in the 134 bp region were similarly bound by hepatic nuclear proteins from hypothyroid, euthyroid, and hyperthyroid rats, except the direct repeat to which the binding of rat hepatic nuclear proteins was dependent on the thyroidal status of the rats (249). Although the obese Zucker rats are generally considered to be in a mild hypothyroid state (69), we did not observe any significant differences between the levels at which the 134 bp DNA fragment was bound by the hepatic nuclear proteins from obese and lean Zucker rats. This may result from the interaction between the nuclear proteins that bind to the direct repeat and those that are positively regulated by glucocorticoids or insulin since obese Zucker rats, as has been mentioned above, are hyperresponsive to the effect of glucocorticoids and have a higher level of hepatic ME mRNA compared with lean Zucker rats (118). It has also been shown that insulin increases the rat hepatic mRNA levels even in the absence of thyroid hormone, whereas the thyroid hormone was not able to increase the hepatic mRNA levels in the absence or low levels of insulin (230).

Although the sequences from +1 to -41 have been shown to be sufficient to initiate expression of the ME gene, the region +1 to -102 is not enough for maximal promoter activity (245). Sequences further upstream are involved in transcriptional regulation of the ME gene by a number of nuclear proteins.

The binding of hepatic nuclear proteins to the 385 bp fragment

The band shift assay performed with the 385 bp DNA fragment yielded four distinct bands (Figure 10).

Located at -103 to -488, the 385 bp DNA fragment contains a number of binding sites for transcription factors and nuclear proteins known to be important in the regulation of transcription activity of a variety of genes (Figure 1, Table 2).

AP-1 binding site

The AP-1 (activator protein-1), initially described as a DNA-binding protein in Hela cell extracts (264), has been shown to be bound by Jun-Jun or Jun-Fos dimers (265). Studies performed by Diamond et al. (266) have demonstrated that the changes in the ratio of Fos to Jun proteins in the AP1 complex could shift the glucocorticoid regulation of the mouse proliferin gene from positive to negative. It has also been shown by other researchers (267) that different cell-specific environments could result in different interactions of the GR with the Jun and Fos proteins, which in turn could manifest different functions.

c-Jun has been shown to possess a glutamine-rich activation domain that could interact with other DNA binding proteins such as the sterol response element binding protein (268). Coincidentally Sp1, as will be discussed below, also contains a glutamine-rich region in its amino-terminal domain which might be involved in the interaction with other transcription factors (269).

Sp1 and IRE-II

As a result of enhanced parasympathetic drive to the pancreatic β cells, insulin secretion is dramatically increased in the obese *fa/fa* rat (46,

106, 270-271). The resulting high insulin level in obese rats might directly increase the binding of certain nuclear proteins to the IREs in many gene promoters. IRE-II in the 385 bp DNA fragment is a positive insulin response element similar to the GAPDH gene promoter IRE-A (224) as has been shown by Garcia-Jimenez et al. (230) It overlaps the Sp1 element in the same region (Figure 1, Table 2). It has been suggested that insulin might enhance the expression of ME gene by interacting with the Sp1 protein and subsequently inducing the binding of the 95 kd and 105 kd Sp1 species (230).

Sp1 is a transcription factor with the unusual property of activating TATA-less promoters (272). The gene for Sp1 was cloned in 1987 (273), a year after it was purified to homogeneity and biochemically characterized (274). The Sp1 element which it recognizes (Table 2) exists singly or multiply in a vast array of cellular and viral promoters (269), including the ME gene promoter used in this study. Sp1 possesses a glutamine-rich region in its amino-terminal domain which may interact with other transcription factors.

In a reconstitution study performed in 1990 (272), Pugh and Tjian provided evidence for the existence of "coactivators" which are required for Sp1 activation of transcription. Moreover, by comparing the TFIID requirement at TATA containing promoters and TATA-less promoters, they were able to demonstrate the presence of an additional "tethering" activity distinct from the above mentioned coactivators. They proposed that the tethering activity might interact with both Sp1 and other transcription factors such as the TATA binding protein and assist the formation of the basal transcription initiation complex at the TATA-less promoters (272).

Although a sequence similar to TATA box has been found at -621 to -626 of the ME gene, whether it really functions as a TATA box is questionable because a TATA box with a position this far from the transcription start site is very rare. Thus it seems more appropriate to classify the ME gene promoter as a TATA-less promoter.

Therefore it is very likely that Sp1 and/or the IRE-II binding proteins (224) might participate in the formation of one or more of the four DNA-protein complexes detected in the band shift assay performed with the 385 bp DNA fragment (Figure 10).

As will be discussed below, binding of hepatic nuclear proteins from obese and lean rats to a 222 bp DNA, which locates just upstream of the 385 bp region, requires proper phosphorylation of the nuclear proteins. Sp1 has also been shown to have the ability to cooperate with the sterol regulatory element-binding protein in sterol regulation of certain genes such as the low density lipoprotein receptor gene (269).

IRE

The regulation of ME gene expression by thyroid hormones has been extensively studied by V. M. Nikodem's group, the same group which conducted molecular cloning and structure characterization of the ME gene (235, 245, 249, 268).

It has been demonstrated that the thyroid hormone increases the level of ME mRNA in rat livers by both transcriptional activation and stabilization of malic enzyme mRNA precursor (275). Concomitantly, a TRE in the ME gene was identified (248) and functionally characterized (276).

The binding to the AP-1 site on the 385 bp DNA fragment by hepatic nuclear proteins from euthyroid rats was higher compared to the hypothyroid rats and much lower if compared to the hyperthyroid rats. As has been mentioned above, the thyroidal status has a similar effect on the binding to the unique direct repeat on the 134 bp DNA fragment by the rat hepatic nuclear proteins (249). This indicates that the thyroid hormone receptors, which are the cellular analogues of the viral *erbA* oncogene (277), might somehow interact with c-Jun protein and the nuclear proteins that bind to the 10 bp direct repeat in the 134 bp fragment. The fact that these three elements lie close to each other supports this view (Figure 1). Moreover, the thyroid hormone receptor has also been shown to interact with components of the transcription initiation machinery such as TFIIB (278) and TATA binding protein (279).

Therefore the four bands shown in lanes 2 and 3 in Figure 10 might represent the DNA-protein complexes formed through the binding to the *cis*-acting elements by the above mentioned transcription factors or nuclear proteins. Whether the DNA-protein complexes are formed by one or more proteins is not clear.

An obvious difference between the 134 bp and 385 bp DNA fragments is that the latter was bound by the hepatic nuclear proteins from obese rats in a greater extent than by those from lean rats (Figure 10), while there was no significant difference in the binding to the other. This might result from increased binding of insulin related factors to the IRE-II/Sp1 site in obese Zucker rats. Other nuclear proteins which positively regulate ME

gene expression might also participate in the formation of these specific DNA-protein complexes.

The binding of hepatic nuclear proteins to the 222 bp fragment

A distinctive feature of the 222 bp DNA fragment is that the patterns in which the hepatic nuclear proteins bind to it are different between obese and lean Zucker rats (Figure 11). This difference could result from differential binding to the 222 bp DNA fragment by a single transcription factor or a combination of interactions among many transcription factors that bind to this or even other regions of the ME gene promoter.

GRE

The 3' end of the 222 bp DNA fragment contains an imperfect GRE (Figure 1, Table 2). Whether this GRE in the ME gene is really bound by glucocorticoid receptor has not been confirmed by experiments. Being a partially palindromic sequence, the GRE can be bound by either monomers or dimers of the glucocorticoid receptor (173-175). The binding to the GRE by the receptor dimers is cooperative (173-175). As has been mentioned in the Review of Literature, the glucocorticoid receptor binds to the GRE with its "zinc fingers" which are located in the DNA binding domain of the GR protein. Many nuclear proteins have been found to have a synergistic effect on the transcriptional activity of the GR. It's suggested that they may serve to "bridge" receptors to the transcription machinery (262). Therefore the GR may participate, through interaction with other nuclear proteins, in the formation of one or more specific DNA-protein complexes observed in the band shift assay performed with the 222 bp DNA fragment.

TATA

The TATA box in the 222 bp DNA fragment does not seem to really function in the initiation complex of transcription since its position is too far from the traditional TATA box, which is always located close to the transcription start site. But the possibility that it might somehow be involved in transcription can not be ruled out because no experiments have been performed to investigate its function.

HNF3 and IRE-I

In contrast to the positive IRE-II at -162 to -169 in the 385 bp DNA fragment, the IRE-I at -693 to -687 in the 222 bp DNA fragment resembles the PEPCK promoter IRE (230), the binding to which by a hepatic nuclear protein has been shown to be negatively regulated by insulin (230). Unlike the PEPCK IRE, which overlaps the AF2 element of a composite GRE unit (230), the IRE-I in the 222 bp DNA fragment is separated from the GRE (Figure 1, Table 2). However, it overlaps a potential binding site for HNF3 proteins (Figure 1, Table 2).

The HNF3 family of proteins were originally identified as transcription factors that bind to promoters and enhancers of several liver specific genes and coordinately regulated their expression (280). Later these proteins were found to play important roles in embryogenesis when body axis formation and commitment to different developmental fates are decided (281). The HNF3 family consists of three proteins (α , β , and γ) which share strong homology in DNA binding domains that bind to the same DNA sequence. There are three more regions that are homologous among them, one in the amino terminus and two in the carboxyl terminus (282). Moreover, HNF3

proteins were also demonstrated to be homologous with the *Drosophila* homeotic gene fork head (282). A possible reason for the existence of three related but different HNF3 proteins is that they could be differentially modified in response to various signals (280). Thus one possible explanation for the observation that the patterns in which hepatic nuclear proteins bind to the 222 bp region are different between obese and lean rats is that the ratios of different HNF3 proteins binding to this region are different between them.

The overlapping of the IRE-I and HNF3 binding site at the 222 region might indicate that the binding by one nuclear protein interferes with that of the other. It is possible that one or more DNA-protein complexes detected in Figure 11 might result from the retro-excluding binding by the HNF3 proteins and IRE-1 binding proteins.

The binding of hepatic nuclear proteins to the 186 bp fragment

Located at -696 to -882, far from the transcription start site, the 186 bp DNA fragment contains no binding sites for known transcription factors or nuclear proteins (Figure 1). However, our results clearly indicated that this region of the ME gene is bound by certain nuclear proteins since at least two specific DNA-proteins complexes were formed when the 186 bp DNA fragment was incubated with the hepatic nuclear proteins from obese and lean Zucker rats (Figure 12).

The levels of binding by the nuclear proteins from obese and lean Zucker rats to the 186 bp DNA fragment are different. However the overall patterns of the binding are the same between obese and lean animals. Therefore this region of the ME enzyme gene might participate in the

regulation of the expression of the ME gene, but it does not seem to function as a primary factor in the pathway of the regulation.

Like other DNA fragments from the 882 bp upstream region of ME gene, the 186 bp DNA fragment was bound at a much higher level by obese hepatic nuclear proteins than by lean ones (Figure 12). This might be a result of interactions among the nuclear proteins that bind the -696 to -882 region and those that bind other regions such as HNF3 binding site, IRE-I and the GRE which are all located relatively nearby (Figure 1).

The binding of hepatic nuclear proteins to the 49 bp fragment

The binding to the 49 bp DNA fragment (-463 to -511), which contains a complete GRE, resulted a major specific DNA-protein complex which is more prominent in obese than in lean rats. Because GRE is the only known *cis*-acting element in the 49 bp region (Table 2), it is very likely that this DNA-protein complex is formed by the binding of GR to the GRE. The higher level of the complex in obese Zucker rats might indicate that obese hepatic nuclear extract contains more GR. This agrees with the report that Western blotting of nuclear protein extract has shown an increase in GR protein in nuclei of obese Zucker rats (261). If this is the case, GR might contribute to the formation of the Complex 1 in Figure 11, which resulted from binding of hepatic nuclear proteins to the 222 bp DNA fragment since that complex is also more prominent in obese rats than in lean rats.

The effect of adrenalectomy

The function of glucocorticoids in the development of obesity in this genetic model of obesity has been investigated in a number of ways in the past few years (46, 90, 106, 115-116, 130). The results of Bray and York

(118) identified an increase in the mRNA levels of the genes for ME and GAPDH in liver and adipose tissue of obese Zucker rats that was dependent upon glucocorticoids. These increases were eliminated by adrenalectomizing the animals and then restored by replacement doses of glucocorticoids. This led to their initial hypothesis that the *fa* gene mutation may result directly or indirectly in a change in the levels or activity of one or more transcription factors that modulate glucocorticoid regulated genes. Such an effect is likely to be expressed in many tissues. While the majority of studies have concentrated on glucocorticoid responses in liver and adipose tissue of obese rats, evidence that there are also alterations in the central responses to the glucocorticoids has been presented from several laboratories (283-284).

The finding that the obese nuclei contain more protein that binds to DNA in the -712 to -491 region of ME gene, which contains an HNF3 binding site, the IRE-I, and an imperfect GRE, and the abolishment of the formation of the DNA-protein complex by adrenalectomy of the Zucker rats (Figure 15), supports the possibility that there may be alterations in transcription factors that modulate glucocorticoid responses in the obese *fa/fa* rat.

Cross-talk between signal transduction pathways has been recognized as a common paradigm in cellular development and homeostasis. Insulin degrading enzyme (IDE) was reported to interact with GR and AR (androgen receptor), which may potential couple insulin and steroid hormone signaling pathways. This relationship may be important for various biological phenomena, such as insulin resistance, and specificity of steroid hormone action (285).

Phosphorylation status

A rapidly growing area of interest in the study of protein phosphorylation is the regulation of cellular growth through phosphorylation of key nuclear proteins (286). These nuclear proteins include some transcription factors such as CREB (287), Jun, Fos (58, 288-289), NF- κ B (289), Myc (289), and Myb (290-291), and tumor suppressor proteins such as pRB (292) and p53 (293-294). Regulation of the activities of these proteins can occur through phosphorylation and dephosphorylation mechanisms that in turn respond to the timing mechanism of the cell cycle, or directly to extracellular signals transduced to the nucleus by "cascade events". In addition, some viral proteins that stimulate cellular growth are also regulated by phosphorylation or are able to influence the phosphorylation of proteins central to growth regulation (285).

All members of the steroid hormone receptor family are phosphoproteins. On incubation of cells or tissues in culture with the respective ligands, steroid receptors become hyperphosphorylated. This extraphosphorylation is a rapid process (<30 min) and is associated with receptor transformation (295-297). But whether the increase in steroid receptor phosphorylation upon hormone treatment is the consequence of a conformational change, making the receptor a better substrate for a kinase(s) or a poorer substrate for a phosphatase(s), is unknown (181).

Our study showed that certain DNA-protein complexes formed by binding of the 222 bp DNA fragment by Zucker rat hepatic nuclear proteins were abolished by dephosphorylation of the nuclear proteins (Figure 16). This clearly indicates the involvement of protein phosphorylation in the modification of the transcription regulation of the ME gene. When the

formation of Complexes 1-3 in Figure 16 were abolished by the dephosphorylation of the nuclear proteins, the formation of Complex 4, was promoted. In fact, at least two more DNA-protein complexes appeared with similar migration rates to Complex 4. It is likely that the nuclear proteins in these small complexes represent those which together, when properly phosphorylated, form the Complexes 1-3 which migrates much more slowly. These proteins can still bind to the DNA but they can no longer interact with each other to form a complicated complex on the DNA fragment.

Recent studies indicate that cellular kinases play a prominent role in the regulation of members of the steroid/thyroid hormone receptor superfamily. PKC (298) and PKA (299) have been implicated to enhance the transcription activation activities of the GR (298). PKA was also reported to increase the receptor mRNA (300).

Transcriptional regulation by cAMP occurs through a multistep process involving activation of cAMP-dependent PKA and subsequent phosphorylation of transcription factors such as cAMP response element binding protein (CREB) or activating transcription factor-1 which activate transcription by binding to the cAMP response elements (CREs) of target gene (301-302). Several genes are coordinately regulated by both glucocorticoids and cAMP (303-306). Moreover, Rangarajan et al. were able to demonstrate that PKA modulates the steroid sensitivity of a target cell by enhancing the DNA binding activity of GR for GRE (299).

The activity of adenylate cyclase is under control of both stimulatory and inhibitory hormone receptors. Receptors for stimulatory hormones are coupled to adenylate cyclase via the stimulatory guanine-nucleotide-binding

protein Gs while the effects of inhibitory receptors are mediated via one or more of the inhibitory G proteins: Gi-1, Gi-2 or Gi-3 (161, 307). A loss of Gi function in hepatocyte membranes in obese Zucker rats but not their lean counterparts has been reported (308-309). Houslay showed that ligands which enhanced $G_{i\alpha-2}$ phosphorylation in intact hepatocytes from lean Zucker rats failed to do so in hepatocytes from obese animals. They suggested that this defect in guanine-nucleotide-mediated "G_i function" seen in obese Zucker rats may be due to an inactivating phosphorylation of αG_{i-2} caused by elevated PKC activity (161).

However, in our experiments, treatment of Zucker hepatic nuclear proteins with PKA failed to enhance the binding of the nuclear proteins to the 222 bp DNA fragment (Figure 17). PKC treatment of the Zucker hepatic nuclear proteins did not increase the binding of the hepatic nuclear proteins to the 222 bp DNA fragment either (Figure 18). In fact it decreased the DNA-protein complex formation. This is not surprising because the *in vivo* phosphorylation of the nuclear proteins is a well regulated, protein specific, amino acid residue specific, and protein kinase specific process. Therefore the phosphorylation of the improper nuclear proteins, the phosphorylation of the right nuclear protein at the improper residues, or over-phosphorylation of the nuclear proteins would decrease the ability of the nuclear proteins to bind to specific DNA sites (310).

Nevertheless, DNA-dependent phosphorylation has been suggested to be a mechanism for transcription activation (311). It has been shown that Sp1 can be quantitatively and efficiently dephosphorylated by protein phosphatases, the activities of which do not require the binding of Sp1 to

DNA and can be inhibited by okadaic acid (312). Thus it is possible that the dephosphorylation of Sp1 might account partly for the disruption of some of the DNA-protein complexes formed by the binding of the hepatic nuclear proteins to the 222 bp DNA fragment.

Different regions of the 222 bp DNA fragment

In diabetic rats (type I), ME mRNA was undetectable but was increased by insulin. The transcription rate of the gene was also reduced in the diabetic liver and recovered after insulin therapy (230). Garcia-Jimenez, et al. found that the protein/IRE-I complex increased in liver nuclear extracts from diabetic rats and decreased after insulin administration, suggesting that this sequence could mediate negative regulation by insulin as has been reported by O'Brien (3, 225). In contrast, the protein/IRE-II complex decreased in liver nuclear extracts from diabetic rats and increased after insulin administration.

In our study the Complex 1 formed by the 222 bp DNA fragment and nuclear proteins increased in obese Zucker rats compared to those from the lean Zucker rats. In order to find out if IRE-I is involved in this increase we used a 70 bp fragment, which contain the overlapping IRE-I and HNF3 binding site, in the band shift assay and the result showed that the binding of nuclear proteins to this region was weak in both obese and lean Zucker rats (Figure 19). This agrees with Garcia-Jimenez's report because the insulin level is not reduced in either the lean or obese Zucker rats. However glucocorticoid replacement on adrenalectomized obese and lean Zucker rats demonstrated a dramatic increase in binding of nuclear proteins to the 70 bp region (Figure 19). We suspect the increase in binding was caused by HNF3

other than the IRE-I binding protein because the replacement with dexamethasone increased the hepatic ME mRNA level in Zucker rats (118); and proteins of the HNF3 family, the binding sites of which were found to overlap IRE, have been shown to be required for the full induction of PEPCK gene transcription by glucocorticoids (246). Moreover, the HNF-3 β protein has been shown to possess two casein kinase I and two casein kinase II phosphorylation sites (313), indicating a putative regulatory role phosphorylation might play.

However the possibility cannot be ruled out that insulin might indirectly regulate the glucocorticoid responsiveness of genes through modulation of transcription factor gene expression, which has been shown to be true for the expression of c-fos (273).

The GRE and IRE/HNF3 binding sites are located fairly close to each other in the upstream region of ME gene. Our finding that the IRE/HNF3 binding site needs the presence of GRE or treatment of the rats with glucocorticoids to form the specific DNA-protein complex intrigue us. We propose that part of the metabolic changes in fa/fa rats might be caused by the improper phosphorylation of one or more nuclear proteins which interact with GR, HNF, and/or insulin related factors to regulate gene expressions.

The 152 bp DNA fragment derived from the 222 bp region generated at least 5 DNA-protein complexes when bound by hepatic nuclear proteins from obese and lean Zucker rats (Figure 20). The formation of Complex 1 by obese rat nuclear extract was much stronger than that by lean nuclear extract. This may due to the presence of the GRE in this region since the

position of this complex is comparable to the position of Complex 1 in Figure 11.

Complexes 1 and 2 in Figure 20 were abolished by the dephosphorylation of the nuclear proteins indicating that the formation of these two complexes require the phosphorylation of the involving proteins. Dephosphorylation of the nuclear proteins seems to inhibit the interactions among DNA binding proteins giving rise to many smaller DNA-protein complexes that contains fewer nuclear proteins or single protein (i.e. Complexes 3-5).

BuGR2

Our study showed that formation of specific DNA-proteins complexes were decreased by the BuGR2 antibody (Figure 21), which is directed against the rat glucocorticoid receptor, indicating that this antibody blocks the binding of glucocorticoid receptor to its cognate sequence and/or the interaction of the GR with other transcription factors. This finding further supports the GR's role in the regulation of the expression of ME gene.

It is not surprising that the BuGR2 antibody blocked the formation of the DNA-protein complex instead of binding to the DNA-protein complex and causing a supershifted band because the BuGR2 antibody "has been shown to react with a single epitope in the DNA-binding domain of the glucocorticoid receptor". The binding of the antibody to GR might also change the conformation of the protein and affect its transactivation activity, which distributes all over the entire GR protein (262).

Anti-HNF3 α antiserum

In contrast to the inhibitory effect of BuGR2 on the binding to the 222 bp DNA fragment, the treatment of the Zucker hepatic nuclear proteins with anti-HNF3 α antiserum actually increased the binding. This increase in binding might be mediated by the interaction of HNF3 α with its antiserum, which might somehow changed the conformation of the HNF3 α protein, rendering it able to bind to the DNA. This cannot be caused by the binding of HNF3 α antiserum to the probe since the antiserum alone did not form any shifted band when incubated with the labeled 222 bp fragment (Data not shown). Future experiments with monoclonal antibodies or antibodies against other HNF3 proteins such as HNF3 β may resolve this question.

Proteins in Complex 1

When the DNA-protein complex 1 (Figure 11) from a scaled up band shift assay were resolved in SDS PAGE, a protein with a M.W. of 123 kd was seen in the complexes formed with both obese and lean hepatic nuclear extracts (lanes 3 and 5 in Figure 23). A 31 kd protein was present in the obese Complex 1 whereas a smear around this M.W. range was seen in the lean Complex 1, probably indicating the presence of several proteins.

An interesting finding is that when the original hepatic nuclear proteins were run on the non-denaturing PAGE, protein-protein complexes that have the same migration rate as Complex 1 were found to contain different proteins between obese and lean rats (lanes 2 and 4 in Figure 23). The protein-protein complex in obese extract has the same components as the DNA-protein Complex 1. In contrast there were clearly one extra protein in

the protein-protein complex in the lean nuclear extract compared to the DNA-protein Complex 1. The M.W. of the this protein is 33 kd.

A possible explanation for the absence of this protein from the DNA-protein complex 1 might be that this small protein interacts with the larger proteins to form protein-protein complexes in the lean nuclear extracts. When the target DNA was available, the small protein may dissociate from the larger proteins and bind to the target DNA to form small DNA-protein complexes such as Complex 4 in Figure 11. The larger proteins also bind to the target DNA but the larger protein themselves have a lower concentration in lean extracts compared with the obese extracts. The possibility that the absence of this 33 kd protein from the obese nuclear extract represent the fa defect is very appealing.

SDS PAGE of the original nuclear extracts

The SDS PAGE of the original nuclear extracts from obese and lean Zucker rats demonstrated a similar overall pattern (Figure 24). No extra bands were seen in either the hepatic nuclear extracts or the lean ones. The concentration of most of the proteins that can be resolved showed an obvious increase in hepatic nuclear extracts from the obese Zucker rats compared to those from the lean ones (Figure 24).

2-D PAGE of the original nuclear extracts

Since its introduction in 1975 by O'Farrell et al. (260), high-resolution two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D-PAGE) of proteins has been used to examine a wide variety of biological systems. It is unmatched by any other technique for simultaneously resolving

hundreds of polypeptides. The application of this method has been documented in thousands of publications (314).

The nucleus, the site of DNA replication and differential gene expression, is one of the major subcellular organelles that are critically involved in cellular proliferation and differentiation (315). The nucleus is an extremely complex structure containing DNA, RNA, and about 5-15% of the total cellular protein according to the particular cell type. In general, nuclear proteins include histone proteins which are relatively basic and generally associated with DNA, and nonhistone proteins which are highly complex and heterogeneous, relatively acidic, and associated with all subnuclear components. The functions of nonhistone proteins range from structural, enzymatic, to gene regulatory functions. The nonhistone proteins include nucleoskeletal proteins (316), RNA splicing and transport proteins, sequence specific DNA-binding proteins (317), hormone and growth factor receptor molecules (318), oncogene and tumor suppressor proteins (319), protein kinases and phosphatases (320), transcriptional activators and repressors (321), coactivators, adaptors, and accessory proteins (322).

With the development of high resolution 2-D PAGE many reports have appeared describing detailed analysis of nuclear associated polypeptides from both normal and transformed cell lines and whole tissue samples. Detailed rat liver epithelial cell nuclear protein database has been established (315). The 2-D PAGE study of differential polypeptide expression in adipose tissue of lean and obese Zucker rats performed by Laurent-Winter et al. showed an increase in the accumulation of several peptides in the adipose tissue of obese rats at 30 days of age (323).

However 2-D PAGE information about the nuclear proteins from Zucker rats has not been reported before our study.

Although the SDS PAGE of the nuclear extracts showed an increase of the concentration of most of the proteins in the obese rats, the result from the 2-D PAGE (Figure 25-26) showed two spots in the hepatic nuclear extracts from the lean Zucker rats. These two proteins have a pI of 5.4 and their M.W. are approximately 31 kd and 32 kd, respectively. The fact that they are N-terminal blocked is not surprising because this happens with most of the eukaryotic nuclear proteins.

Since the *fa/fa* rats are supposed to have a defect in the function of the receptor for the *ob* gene product, it is possible that the absence of the two proteins in the *fa/fa* nuclear extracts might represent the impairment of the receptor for the *ob* gene product.

Interestingly, the difference between the M.W. of these two proteins (31 kd and 32 kd) and that of the small protein in the protein-protein complex with the same migration rate as the DNA-protein Complex 1's (33 kd) is practically ignorable because these data were obtained from different PAGE with different molecular weight standard and measuring errors can easily cause such a difference. Thus, the 33 kd protein absent from obese nuclei is like the same as the 31 and 32 kd proteins shown absent from the 2-D PAGE. Future investigation needs to be conducted to further characterize these proteins.

SUMMARY

The binding of proteins from hepatic nuclear extracts of lean and obese Zucker rats to the upstream region of the gene for one of the glucocorticoid-dependent enzymes has been tested using band shift assays. DNA fragments from the upstream region of the malic enzyme gene were used as target DNAs. Nuclear extracts were prepared from young lean and obese Zucker rats (4-6 weeks old).

An increase in binding of nuclear proteins to the upstream region of ME gene was observed in nuclear extracts from obese rats. The specificity of the binding was demonstrated by adding excess amount of either unlabeled target DNA or unrelated DNA in the binding reaction.

A 222 bp DNA fragment (-712 to -491) from the upstream region of ME gene was found to be bound in different patterns by the hepatic nuclear proteins from obese and lean Zucker rats. A large DNA-protein complex was found to be more prominent in obese rats than in lean rats, while other DNA-protein complexes were more prominent in lean rats than in obese rats.

Adrenalectomy reduced a specific DNA-protein complex formation and glucocorticoid replacement restored the specific DNA-protein complex formation in both lean and obese rats.

Alkaline phosphatase treatment of the nuclear proteins reduced the specific DNA-protein complex formed with the 222 bp DNA of ME gene in both obese and lean rats.

A monoclonal antibody against glucocorticoid receptor was found to interfere with the formation of a large specific DNA-protein complex when the 222 bp DNA fragment was used.

2-D PAGE of the nuclear proteins from obese and lean rats showed that there were two proteins present in lean nuclear extract but absent from obese nuclear extract.

CONCLUSION AND PERSPECTIVES

Differences in nuclear proteins binding to the upstream region of ME gene between lean and obese Zucker rats may reflect changes in phosphorylation state of the nuclear proteins. Glucocorticoid receptor seems to be involved in the regulation of the phosphorylation status of the nuclear proteins.

Cloning of the Fa gene will provide invaluable information in the obesity study. However, how the product of Fa gene interacts with the signal transduction pathways and regulates gene expression needs to be clarified before the mechanisms of the development of obesity syndrome can be fully understood.

The purification and characterization of the two nuclear proteins in lean Zucker rats that are absent from obese Zucker rats may provide insights into the influence of the Fa gene product on the composition and functions of the nuclear proteins.

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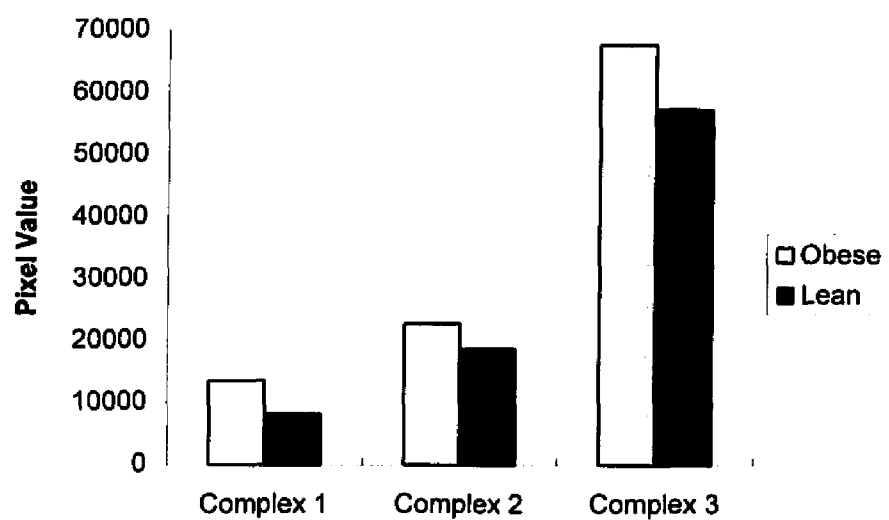
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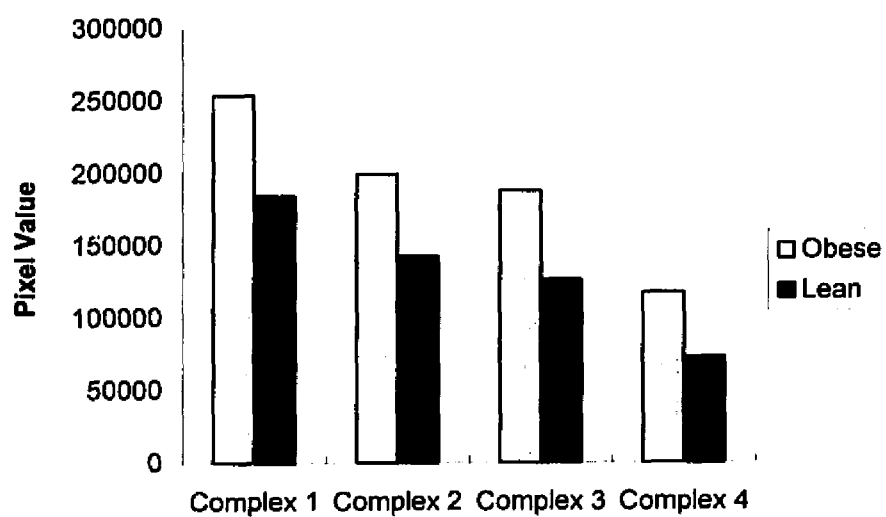
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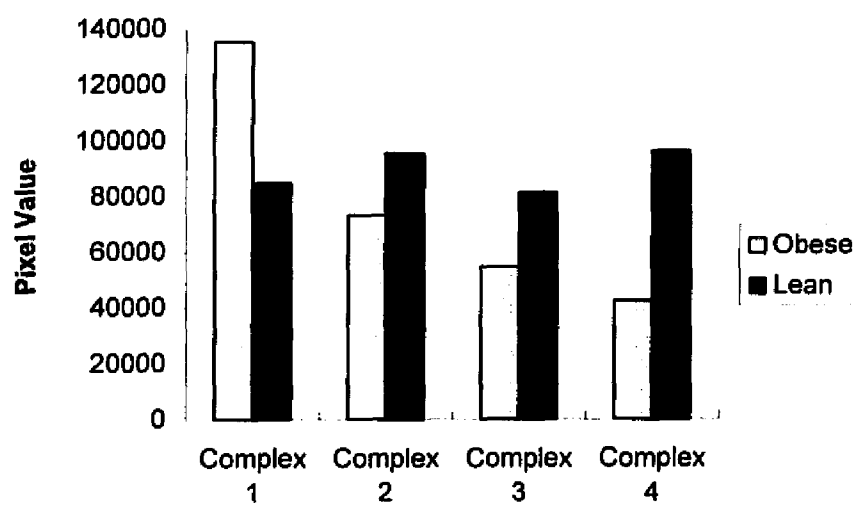
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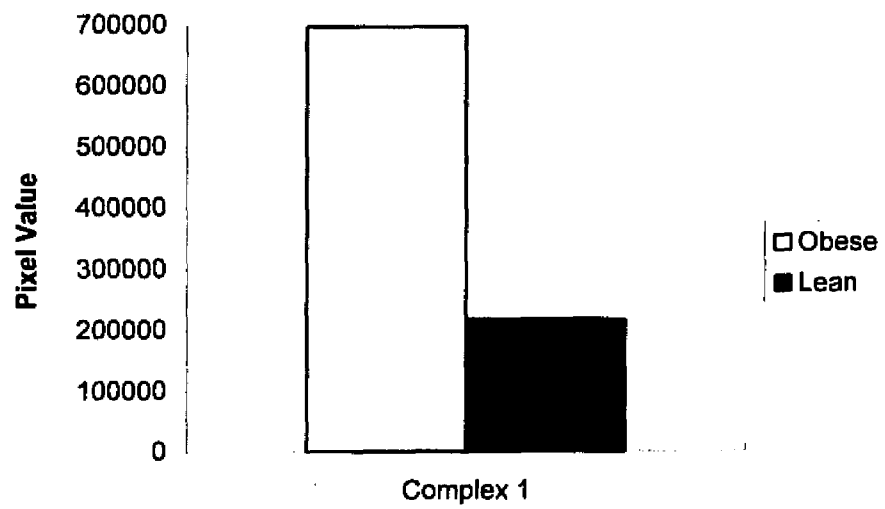
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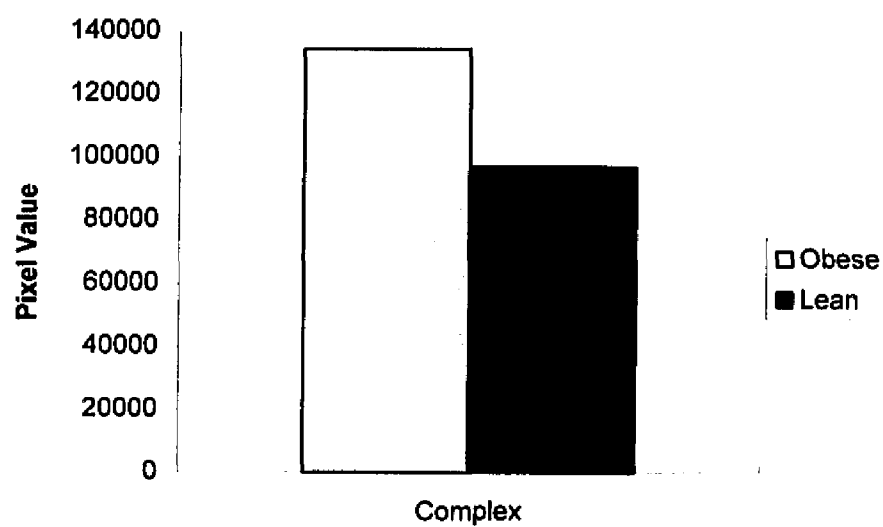
APPENDIXES

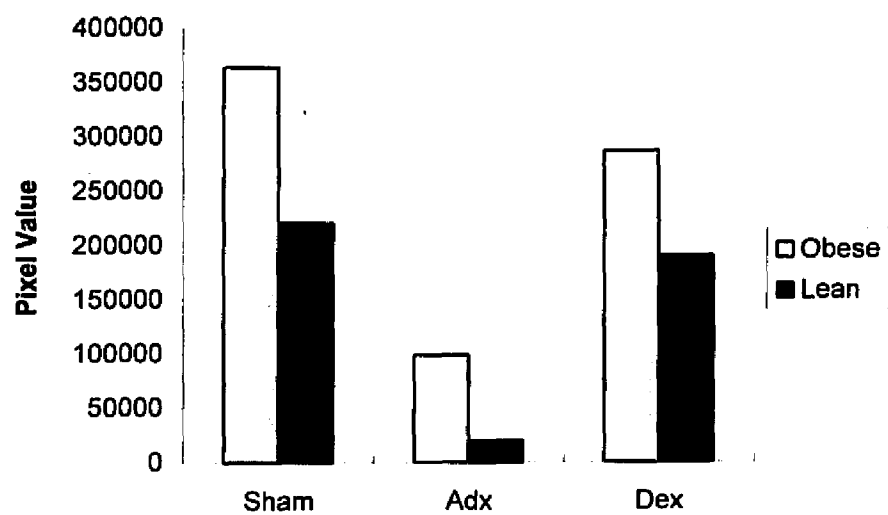
A. Quantitation of Complex 1-3 in lanes 2-3 in figure 9

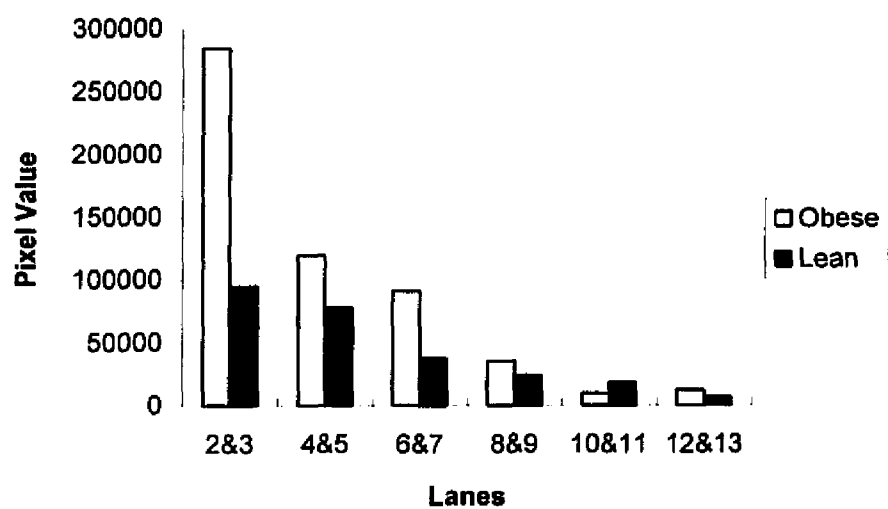
B. Quantitation of Complex 1-4 in lanes 2-3 in figure 10

C. Quantitation of Complex 1-4 in lanes 2-3 in figure 11

D. Quantitation of Complex 1 in lanes 2-3 in figure 12

E. Quantitation of the DNA-protein complexes in lanes 2-3 in figure 14

F. Quantitation of Complex 1 in lanes 2-7 in figure 15

G. Quantitation of Complex 1 in lanes 2-13 in figure 16

VITA

Yibing Wang was born on March 3, 1965 in Jinan, Shandong, China. After graduating from Shandong Experimental Middle School in 1982, he attended Shandong Medical University College of Medicine where he obtained his M.D. in 1987. Then he began graduate study in Shandong Medical University with a Cardiology Fellowship from the Shandong Academy of Medical Sciences. At the same time he received his residency training in the Department of Cardiology at the Affiliated Hospital of Shandong Medical University. He graduated in 1990 with a Master in Medicine. He was then enrolled in the doctoral program in the Department of Microbiology at Louisiana State University in 1990. He pursued a graduate degree in Genetics under the direction of Dr. Braymer and upon completion of his degree requirements will receive a Ph.D. degree in the Department of Microbiology majoring in Genetics, minoring in Biochemistry.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

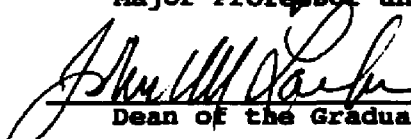
Candidate: Yibing Wang

Major Field: Microbiology

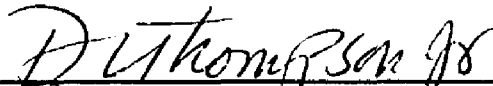
Title of Dissertation: Binding of Hepatic Nuclear Proteins from Obese and Lean Zucker Rats to the Upstream Region of Malic Enzyme Gene


Approved:


Major Professor and Chairman

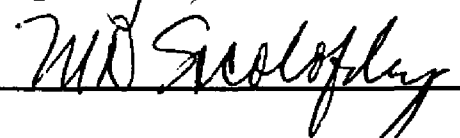

Dean of the Graduate School

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D. L. Thompson


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Date of Examination:

September 19, 1995